EFFECTS OF LIGHT INTENSITY, DAY LENGTH, TEMPERATURE, AND OTHER ENVIRONMENTAL FACTORS ON THE ASCORBIC ACID CONTENT OF TOMATOES

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Variations in the ascorbic acid content of ripe tomatoes are known to be large (Hamner and Maynard, '42). The review of the literature revealed, however, that the data were highly conflicting as to the factors responsible for the variations. It was evident that the majority of the studies had not been sufficiently well controlled to determine the effects of specific factors and their possible interrelations. Therefore, a study was begun in this laboratory 4 years ago in the hope of obtaining specific information which could be utilized in practice to provide consumers with tomatoes of a higher and more reliable nutritional quality. This study has concentrated on environmental factors. Most of the work has been done with an inbred strain of Bonny Best tomatoes and has stressed the use of carefully established conditions so that as many environmental factors as possible were under control.

A study of the variations in carotene content of tomatoes has appeared in a separate report (Ellis and Hamner, '43). In the present report, results are presented of a series of experiments, some of which involve field grown plants and others which were conducted under controlled conditions. The work demonstrates that very large variations in ascorbic acid content of tomatoes may be associated with growing conditions and indicates that a factor of primary importance in determining the ascorbic acid level may be the light intensity a few days previous to harvest.

Unless otherwise stated, the fruits were analyzed on the day that they became red over the entire surface. The methods used in sampling and analyzing were those previously described (Hamner et al., '42). Many individual fruits were analyzed in obtaining each mean value, and the number of fruits, together with the standard error, is presented. All

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values are presented in milligrams of ascorbic acid per 100 gm. of fresh fruit.

Variations in fruit from different locations

In previous studies (Hamner et al., '42), it was found that plants of an inbred strain of Bonny Best tomatoes, grown at five widely separated locations in the United States, produced fruit which differed markedly in ascorbic acid content. These variations in ascorbic acid content, associated with location, could not be correlated with soil conditions or cultural practices, and presumably were related to climatic differences from one location to another.

Because of the results just mentioned, fruits from many locations were analyzed in order to determine the magnitude of the variations in ascorbic acid which might be associated with climatic conditions during the growing season. Since all our experiments with tomatoes have indicated that soil variables produce relatively little effect upon ascorbic acid, fruit from plants grown in soil under the cultural practices prevalent at a given location was obtained for analyses. The fruits were picked when ripe, carefully packed in paper, and shipped by express to Ithaca, New York, for analysis.[1]

The fruits were analyzed as soon as they arrived at Ithaca. While some lots of fruit were doubtless picked at more advanced stages of ripeness than others, and some were a longer time en route than others, these factors were probably not of great importance in determining the differences in ascorbic acid values obtained. Work of others (as reviewed by Hamner and Maynard, '42), as well as work described later in this report, indicates that the changes in ascorbic acid content during storage for a week at room temperature and during the later stages of ripening are not great unless the fruits are obviously "over-ripe". All of these samples were firm and at the "red-ripe" stage.

The result of the analyses are given in table 1. Variations in ascorbic acid content are large even for the same variety. Fruits of Marglobe varied from 14.4 ± 2.12 to 30.6 ± 2.04; of Rutgers, from 8.4 ± 0.50 to 19.7 ± 1.17; of Pritchard, from 10.7 ± 0.88 to 29.0 ± 1.29. Of the two varieties, Marglobe and Rutgers, the former consistently had more ascorbic acid at any particular location, except at Madison, Wisconsin. Both Pritchard and Marglobe were available at only three locations. At two, Pritchard had about 30% less ascorbic acid, and at one the values were about the same. At two locations, Pritchard and Rutgers had about the same ascorbic acid values.

[1] Grateful acknowledgment is made to numerous individuals who cooperated in supplying these fruits.

The results of the analyses are given in table 1. Variations in ascorbic 30.6 ± 2.04. While the nature of the experiment is such that the exact cause of these variations cannot be ascertained, it seems probable that environmental differences among locations were more important than varietal differences.

TABLE 1

Ascorbic acid content of tomatoes raised in various parts of the United States during the summer of 1942.[1]

LOCATION	DATE ANALYZED	VARIETY					
		Marglobe		Rutgers		Pritchard	
		Mean ± S.E.	No. of analyses	Mean ± S.E.	No. of analyses	Mean ± S.E.	No. of analyses
Madison, Wis.	9/11	17.2 ± 0.76	14	19.0 ± 1.09	13	18.9 ± 1.52	14
Kingston, R. I.	8/29	15.5 ± 0.75	15	8.4 ± 0.50	6	10.7 ± 0.88	11
Holgate, Ohio	9/1	22.3 ± 0.90	13	19.4 ± 0.90	12	.....	..
East Lansing, Mich.	9/1	16.7 ± 0.67	15	14.2 ± 0.55	15	.....	..
State College, Pa.	8/31	15.7 ± 0.63	15	14.5 ± 0.70	13	.....	..
Ames, Iowa	9/4	18.4 ± 1.20	14	15.6 ± 0.60	13	.....	..
St. Paul, Minn.	9/8	19.5 ± 1.30	10	.....	..	14.1 ± 0.78	14
Fresno, Calif.	9/8	.....	..	.....	..	29.0 ± 1.29 [2]	10
Orono, Maine	9/21	.....	..	.....	..	19.8 ± 1.13	16
Knoxville, Tenn.	8/29	14.4 ± 2.12	3	.....	..	.....	..
Wenatchee, Wash.	9/1	30.6 ± 2.04	12	.....	..	.....	..
Auburn, Alabama	9/2	.....	..	18.8 ± 1.50	9	.....	..
Charleston, S. C.	9/11	18.9 ± 1.12	10	.....	..	.....	..
Lafayette, Ind.	9/4	.....	..	19.7 ± 1.17	11	.....	..

[1] The following results were obtained with various other varieties at different locations:

Location	*Variety*	*Date*	*No. analyzed*	*Mean ± S.E.*
Riverside, Calif.	Stone	9/1	14	20.9 ± 0.81
Riverside, Calif.	Pearson	9/1	15	30.2 ± 0.82
Mt. Carmel, Conn.	John Baer	8/29	16	13.5 ± 0.60
Davis, Calif.	Pearson	8/25	20	16.0 ± 0.67
Davis, Calif.	Santa Clara Canner	8/25	19	22.1 ± 1.13
Lafayette, Ind.	Ind. Baltimore	9/4	5	19.7 ± 1.61

[2] Twelve fruits were picked when partially ripe and gave values averaging 26.8 ± 1.27.

Degree of ripeness and length of storage

The Pritchard variety, grown in experimental gardens of the Department of Vegetable Crops, Cornell University, were used in these experiments.[2] All fruits were picked September 2, 1940. Fruits at various stages of ripeness were picked as follows: 8 red, soft and obviously "over-ripe"; 20 sound, firm, "red-ripe"; 20 partially red; 400

[2] Acknowledgment is made of the courtesy of Dr. H. C. Thompson for supplying these fruits.

"mature green" (large size, light green with some areas of the surface almost white); 20 "immature green" (about half the size of average ripe fruit). All fruits were analyzed immediately except for 380 of the "mature green" fruits which were used in the storage experiment. These were divided into five lots. A lot of 50 was placed at each of the following storage temperatures: 65°, 70°, and 90°F. An additional lot was placed in storage at 75°F. At each of the storage temperatures, the fruits were analyzed as soon as they turned red over the entire surface. At the higher temperatures some did not turn red, but rather changed from a green to a yellowish pink. These were analyzed when they had lost most of their green color. After 10 days of storage, ten fruits stored at 75°F. were analyzed which were at the "pink" stage, and an additional ten which were still green (table 2).

TABLE 2

Ascorbic acid content of fruit at different stages of ripeness and storage at different temperatures.

TREATMENT	STAGE OF RIPENESS	NO. OF ANALYSES	ASCORBIC ACID MG./100 GM. FRESH WEIGHT	TREATMENT	STAGE OF RIPENESS	NO. OF ANALYSES	ASCORBIC ACID MG./100 GM. FRESH WEIGHT
Initial harvest	Immature green	20	14.0 ± 0.51	*Stored at:*			
	Mature green	20	15.0 ± 0.69	75°—1 week	Red-ripe	11	17.3 ± 1.11
	Pink	20	15.4 ± 0.45	2 weeks	Red-ripe	52	12.9 ± 0.36
	Red-ripe	20	16.2 ± 0.64	3 weeks	Red-ripe	23	7.6 ± 0.38
	Over-ripe	8	16.6 ± 0.91	80°—1 week	Red-ripe	7	14.0 ± 1.58
Stored at:				2 weeks	Red-ripe	25	9.8 ± 0.99
65°—1 week	Red-ripe	4	14.5 ± 2.11	3 weeks	Red-ripe	13	7.1 ± 0.48
2 weeks	Red-ripe	12	15.0 ± 1.78	90°—1 week	Ripe [1]	5	14.0 ± 0.48
3 weeks	Red-ripe	7	8.5 ± 0.39	2 weeks		19	10.8 ± 0.68
70°—1 week	Red-ripe	6	14.4 ± 1.35	75° and removed after	Mature green	10	8.8 ± 0.47
2 weeks	Red-ripe	18	12.9 ± 0.66	10 days	Pink	8	13.1 ± 1.30
3 weeks	Red-ripe	10	8.2 ± 0.75	storage	Red-ripe	17	14.5 ± 0.50

[1] Did not become red-ripe. Analyzed when yellowish pink.

At the time of the initial harvest, the green fruits were only slightly lower in ascorbic acid than the ripe ones. Those which ripened during the first week of storage were approximately as rich in the vitamin as those which were ripe at the initial harvest. Fruits ripening during the second week of storage tended to be a little lower in ascorbic acid, especially at the higher storage temperatures, and those ripening subsequent to 2 weeks of storage, regardless of storage temperature, were appreciably lower in ascorbic acid, and the variability between fruits

was much less. After 10 days of storage at 75°F., the degree of ripeness was correlated with marked differences in ascorbic acid, green fruit having values appreciably lower than ripe fruit. Perhaps those individual green fruits which were low in ascorbic acid at the time of the initial harvest were the same ones which ripened last in storage and thus account for some of the apparent decrease during storage. It is also possible that ascorbic acid was destroyed more rapidly during storage in those which were the "greenest" upon entering storage.

Variety and fertilizer treatment

In previous reports (Hamner et al., '42; Lyon et al., '43, '44) the influence of mineral nutrition on ascorbic acid content was found to be of relatively minor importance. While certain levels of supply of some of the elements did affect the ascorbic acid content, the concomitant effects on growth and fruitfulness were so great that it is doubtful if similar levels of supply would occur under field conditions of commercial production.

During the summer of 1942, analyses were made of three varieties grown at the New York State Experiment Station, Geneva, New York. The details of the experimental design, fertilizer treatments, growth and yield data, etc., will be reported by Dr. C. B. Sayre in a separate publication. There were seven fertilizer treatments with three replications of each treatment for each variety, and seven fruits were analyzed from each replicate. The mean values for ascorbic acid content of each treatment are given in table 3. The significant variation seems to

TABLE 3

Ascorbic acid content of tomatoes produced with plants grown at Geneva, New York, on soil receiving seven different fertilizer treatments.

FERTILIZER APPLICATIONS [1]		VARIETY (ASCORBIC ACID IN MG./100 GM. FRESH FRUIT)			
Kind	lbs./acre	Stokesdale	Rutgers	N. Y. State	Treatment Mean
None	None	22.2 ± 0.99	20.8 ± 0.65	20.7 ± 0.68	21.2
0-20-10	600	24.5 ± 0.78	20.9 ± 0.67	23.5 ± 1.10	23.0
5-20-10	600	22.3 ± 1.33	23.0 ± 0.85	21.6 ± 0.58	22.3
20-20-10	600	18.7 ± 0.67	18.0 ± 0.46	19.6 ± 1.55	18.8
5-20-10 [2]	600	21.6 ± 0.86	21.2 ± 0.70	21.0 ± 0.84	21.3
5-20-20	600	19.9 ± 0.70	19.9 ± 0.65	20.3 ± 0.57	20.0
3-12- 6 [3]	1000	21.2 ± 0.84	20.5 ± 0.46	21.8 ± 0.85	21.2
Varietal mean		21.5	20.6	21.2	

[1] Figures show percentages of available N, P, and K as total N, P_2O_5, and K_2O obtained by analysis of fertilizer.

[2] Sixty pounds of MgO as $MgSO_4$ were also added in this treatment.

[3] Premium grade.

be associated with a heavy application of nitrogen (20-20-10) which resulted in low ascorbic acid values. The differences between this treatment and the others were not great and were probably of little applied significance. In light of the results of an experiment described subsequently, it seems possible that these low values may have been associated with the influence of the heavy nitrogen application in producing greater foliage and resultant shading of the fruit.

Temperature and humidity

In this experiment, use was made of three control chambers previously described (Hamner, '44). The respective temperature and humidity of the chambers was: chamber 1, 78° ± 2°F. and 90 ± 5%; chamber 2, 63° ± 2°F. and 84 ± 5%; chamber 3, 78° ± 2°F. and 30 to 50% relative humidity. Thus, chambers 1 and 2 were operated at two different temperatures with approximately the same vapor pressure deficit (equivalent to 2.3 mm. of mercury). Chambers 1 and 3 were operated at the same temperature with a relatively high and a relatively low humidity, respectively. The chambers were illuminated for 16 hours each day with "white" and "daylight" fluorescent lamps being used alternately (Hamner, '44), the illumination intensities as measured by a Weston foot-candle meter being from 800 to 900 foot-candles. The various environmental factors, other than those under study, were controlled as nearly alike as possible.

Seedlings of the inbred strain of Bonny Best tomatoes were germinated in sand in the greenhouse and the seedlings transplanted singly into two-gallon crocks containing quartz sand. Eight plants were placed in each chamber, and eight more plants were continued on experiment in the greenhouse. The plants were watered three times each week with a balanced nutrient solution. Each week the crocks were thoroughly flushed with distilled water and nutrient solution applied immediately thereafter. The plants grew most rapidly at high temperature and humidity (chamber 1) and nearly as rapidly at high temperature and variable humidity (chamber 3), whereas in chamber 2 growth was much slower. The tops of the plants were supported with string in order that the foliage of each might receive as much light as possible. The plants were not pruned in any way. The differences between the plants in chamber 1 and chamber 3 were never very great, although the plants of chamber 1 had slightly longer internodes and somewhat longer leaves than those of chamber 3. The plants of chamber 3 more closely resembled comparable plants grown in the greenhouse than did plants of the other two chambers. The plants in chamber 2 grew much

more slowly, produced short internodes and relatively large leaves. Growth of axillary buds was abundant, producing a plant with many branches and a bush appearance. Since these plants were used subsequently in another experiment no data are available as to dry weight accumulation and fruit weight.

Fruiting occurred at a much later date in chamber 2 than in the other two chambers, but the number of fruits set was larger. The fruits produced in the low temperature chamber were small and irregularly shaped (Watts, '31), whereas those at the higher temperatures were relatively small as compared to fruits produced in the greenhouse or out-of-doors, but were regular in shape. No differences were noted in the amount or appearance of fruits from chambers 1 and 3. The fruits were harvested on the morning of the day that they became red over the entire surface.

Ascorbic acid analyses were made on individual fruits. The results of the ascorbic acid analyses in milligrams per 100 gm. fresh weight were as follows: chamber 1, 18.5 ± 0.30 ($n = 29$); chamber 2, 15.6 ± 0.54 ($n = 34$); chamber 3, 19.4 ± 0.39 ($n = 11$); and from the greenhouse plants, 21.5 ± 0.35 ($n = 24$). The temperatures used in this experiment were fairly extreme. A continuous temperature of 63°F. is just about as low as tomato plants can stand to grow and still produce a crop, while a continuous temperature of 78°F. is approaching the upper temperature limits in which plants may be grown successfully. The ascorbic acid values at 63°F. (chamber 2) are significantly lower than those at 78°F. (chambers 1 and 3), although the differences are not great. The plants grown in the greenhouse produced fruit higher in ascorbic acid than in any of the chambers.

Length of photoperiod

Tomato plants of the Bonny Best inbred strain were grown in two of the control chambers in a manner very similar to that described in the previous section. The temperature of the two chambers was maintained at $73° \pm 2°$F., and the relative humidity varied from 60 to 85%. One chamber was illuminated for 8 hours each day and the other chamber for 16 hours. The experiment was conducted from April to July, and comparable plants were grown in the greenhouse, exposed to the natural variations in day length. The results of the ascorbic acid analyses in milligrams per 100 gm. fresh weight of fruit were as follows: chamber 1, 8-hour photoperiod, 16.7 ± 0.28 ($n = 53$); chamber 2, 16-hour photoperiod, 19.5 ± 0.33 ($n = 41$); greenhouse, 22.0 ± 0.37 ($n = 59$). The plants on the 8-hour photoperiod were significantly

lower in ascorbic acid than those grown in the 16-hour photoperiod, whereas the plants grown in the greenhouse produced fruit appreciably higher in ascorbic acid than those from either of the chambers.

Sunshine and shade

On May 4, 1943, seeds of the Bonny Best inbred strain of tomatoes were planted in sand contained in small crocks (about five seeds per pot) and watered thrice weekly with a balanced nutrient solution. Once each week the pots were flooded with distilled water. Shortly after germination, the seedlings were thinned so as to leave two uniform seedlings per crock. On May 28, the seedlings were transplanted to quartz sand contained in 2-gallon crocks, one seedling per crock. On June 19, forty plants were placed outdoors in the sunshine and divided into groups of eight plants each. The individual plants of the various groups were randomized as to position.

One group of plants, designated as the sunshine control plants, remained in the sunshine throughout the rest of the experiment. The second group was grown in sunshine until the first blossoms appeared on June 22–23. These were tagged, and all unopened flower buds in that cluster were removed. The plants were then shaded with cheese cloth until the fruits ripened and were analyzed. The cheese cloth shade reduced illumination by 75% during bright days, as measured with a Weston foot-candle meter. The third group remained in sunshine until July 8, at which time each plant had three or four fruits, approximately ¾ of an inch in diameter. These were tagged; all other fruits and blossoms in that particular cluster were removed, and the plants placed in the shade where they remained until the fruits ripened and were analyzed. The fourth group was transferred to the shade on July 14, when each plant had three or four fruits approximately half grown, i.e., from 1 to 2 inches in diameter. The fifth group remained in the sunshine until each plant had developed from one to four "mature green" fruits, that is, of full size and pale green without any indication of red. Four plants of this group were transferred to shade on July 20, and four others on July 26.

A second lot of plants was handled in much the same way as that described above with respect to planting and cultural procedures. The seeds were planted on May 20 in the small crocks and transplanted to the larger crocks on June 10. On June 19, the plants were placed outdoors and exposed to full sunshine for 3 days. On June 22, when the plants were approximately 4 to 5 inches high, all plants were placed in the shaded area. Forty plants were used, and these were divided into

five groups of eight plants each. The various groups were comparable to those previously described, except that the plants were transferred from shade to full sunlight at stages comparable to the transfer of the previous groups from sunshine to shade.

An outline of the treatments and vitamin analyses is given in table 4. Of the control plants, those grown throughout the experimental period with full exposure to sunshine produced fruits with an average ascorbic acid content of 25.8 ± 0.63 mg. per 100 gm. fresh weight, while those grown in shade produced fruits with an average ascorbic acid content of

TABLE 4

Ascorbic acid content of tomato fruit from plants transferred from shade to sunlight and vice versa at various stages of fruit development.

STAGE OF FRUIT DEVELOPMENT AT TIME THE PLANTS WERE TRANSFERRED	TREATMENT OF PLANTS			
	Grown in sunshine [1]		Grown in shade [2]	
	No. of fruit analyzed	Ascorbic acid content in mg. per 100 gm. fresh wt.	No. of fruit analyzed	Ascorbic acid content in mg. per 100 gm. fresh wt.
Controls; not transferred	56	25.8 ± 0.63	48	15.5 ± 0.43
	Transferred to shade		Transferred to sunshine	
Transferred at time of first blossom	17	17.0 ± 0.88	27	26.1 ± 0.83
Transferred at time first fruits were ¼ in. in diameter	13	17.3 ± 1.75	19	27.0 ± 0.78
Transferred at time first fruits were ½ in. in diameter	27	15.8 ± 0.88	23	26.2 ± 0.68
Transferred at time first fruits were "mature green"	23	16.8 ± 0.72	17	23.4 ± 1.08

[1] Plants were placed outdoors with full exposure to summer sunshine.

[2] Plants were placed under a cheese-cloth shade which resulted in decrease in illumination of about 75% during bright summer days (as measured with a Weston foot-candle meter).

15.5 ± 0.43 mg. The plants which were transferred from sunshine to shade produced fruits with an average ascorbic acid content not significantly different from the shade controls, regardless of the stage of development at the time of the transfer. The plants which were transferred from shade to sunshine produced fruit with an average ascorbic acid content not significantly different from the sunshine controls.

It is apparent that the production of ripe fruit under shade has resulted in a considerable decrease in ascorbic acid content in comparison with fruit produced by plants growing in sunshine. The effect of shading seems to be produced during the period just before the fruits ripen.

No transfers were made at the time some of the fruits were partially colored, and therefore there was no measure of the rapidity with which a changing environment influences the ascorbic acid content during ripening.

DISCUSSION

The present study has dealt with the specific factors of possible significance in the variations in ascorbic acid content of tomatoes grown at different locations. Neither the nature of the soil nor fertilization has been found to exert any marked effect. There is also little evidence in this work that the location effects can be ascribed to differences in temperature, humidity, or variations in day length. The two temperatures used were fairly extreme. Plants growing in the field are subjected to temperature variations that are much more extreme but not continuous. It would seem that if temperature variations had a marked effect upon the ascorbic acid content of field-grown plants, greater differences would have been noted in the results of this experiment. It is possible that rapid fluctuations in temperature might have greater effects upon ascorbic acid values than continuous treatment with a low or a high temperature. The two day-lengths used were sufficiently different to warrant the expectation that much greater differences would not be observed under the natural variations in day length which occur from season to season. However, the illumination intensities used in the experimental chambers were relatively low, and it may be that at higher intensities, greater differences in ascorbic acid content would have been associated with the two day-length treatments. The two relative humidities used were sufficiently different to result in effects upon growth, but produced no effects upon ascorbic acid content. While much greater differences in humidity occur under field conditions, it seems likely that, were humidity playing a dominant role in determining ascorbic acid values, there would have been some differences in this experiment.

The one variable so far studied which seems to exercise marked effects is light intensity. Variations in ascorbic acid content associated with differences in this factor were relatively great. Furthermore, shifting the plants from one light intensity to another produced rapid changes in the ascorbic acid content of the fruit. It seems possible that the intensity of illumination may be the dominant factor in determining ascorbic acid values under many conditions.

Reports (Jones et al., '44) that nitrogen fertilization decreases ascorbic acid content of fruits seem conclusive. Our evidence indicates that this nitrogen effect may be an indirect one. It was found here that

fertilization with nitrogen caused a decrease in ascorbic acid content of fruit from field-grown plants. In previous studies (Hamner et al., '42), no influence of nitrogen supply to tomato plants grown outside in sand culture was found. In the latter case, the plants were grown outside in sand cultures, the vines were supported on poles, and the axillary growth was removed. The pots were about 4 feet apart so that the individual plants were evenly illuminated by direct sunshine. In the fertilizer experiment reported here in which nitrogen fertilization decreased ascorbic acid content, the plants were grown in the field in rows 3 feet apart, and the plants were not pruned in any way. Thus, the heavy application of nitrogen in the field experiment undoubtedly produced more foliage, with resultant increased shading of some leaves by others. In the sand culture experiment on the other hand, while greater growth and more foliage were produced in the cultures supplied with more nitrogen, the design of the experiment was such that increased foliation did not cause increased shading. Thus, one might explain the effect of nitrogen in decreasing ascorbic acid content in one experiment and not in another.

Reports in the literature give abundant supporting evidence of the influence of light intensity on ascorbic acid content (Hamner and Maynard, '42; Maynard and Beeson, '43). This evidence includes reports that the amount of ascorbic acid in individual fruit on one tree may be proportional to the amount of light received during ripening. Cloudy weather has been reported to decrease ascorbic acid, and the side of individual fruit exposed to the sun has been found richer in ascorbic acid than the shaded side. There has been disagreement as to whether or not greenhouse-grown tomato fruits contain less ascorbic acid than field grown fruits, and it may be possible that those investigators who recorded the decrease in greenhouse-grown plants were utilizing greenhouses in which the intensity of illumination was appreciably lower than outdoors. In previous work from this laboratory (Hamner et al., '42), no difference between greenhouse and field-grown fruit was noted, but the plants grown in the greenhouse were exposed to intensities of illumination at least 90% of that outdoors.

Thus, the assumption that light intensity previous to harvest plays the dominant role in determining ascorbic acid levels in tomato fruit of a given variety does not seem to conflict with any of the other experimental data. It seems possible that much of the effect ascribed to season and location may be due to variations in this factor. Since this work indicated that rapid change in ascorbic acid takes place following a change in intensity of illumination, it would seem desirable to harvest fruit after a few days of bright sunny weather if maximum ascorbic acid

values are to be obtained. It may be possible that production of fruit for canning purposes in regions where light intensity is consistently high would result in more ascorbic acid in the canned product, assuming, of course, that the processing methods preserved most of the ascorbic acid present in the fresh fruit. The results of this work emphasize the importance of considering illumination intensities in interpreting the results of experimentation dealing with factors influencing ascorbic acid content of plants.

SUMMARY

The influence of environmental variables on the ascorbic acid content of tomatoes has been studied in the field and under controlled conditions in the laboratory. Evidence is presented that ascorbic acid content is only slightly, if at all, influenced by: (1) degree of ripeness after the fruit is "mature green", (2) storage of the fruit for 10 to 14 days at temperatures from 65°F. to 90°F., (3) fertilizer treatment of the plants (although nitrogen fertilization may cause a slight decrease), and (4) relative humidity to which the plants are exposed.

Under controlled growing conditions fruits produced under lowered temperatures and shortened day length were somewhat lower in ascorbic acid.

By far the greatest influence on ascorbic acid content was produced by variations in light intensity previous to harvest. Increases in the ripe fruit of 66% in ascorbic acid resulted when plants were transferred from shade to sunshine at the time the fruit was mature green. A discussion is given of the possible significance of these results with the suggestion that the light intensity to which the plants are exposed just previous to harvest may be the dominant factor in determining the ascorbic acid content of ripe fruit.

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SHORT PERIOD BLOOD SUGAR TIME CURVES FOLLOWING INGESTION OF SUCROSE

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There is a widespread impression that, for immediate muscular work, the type of sugar is important; that dextrose is a better source of energy for this purpose than sucrose since, unlike the latter, it does not require digestion before it is absorbed from the alimentary tract. Many years ago, however, Higgins ('16) demonstrated the rapidity with which oral administration of sucrose may increase the respiratory quotient, as compared with dextrose; whereas, sucrose produced a very marked rise as early as 4 minutes, a definite rise was not noted with dextrose until a lapse of 20 minutes. The effects of other sugars and of alcohol were also investigated and, from these data, Higgins concluded that sucrose begins to be burned "quite as soon as alcohol". That sucrose affects the respiratory quotient more rapidly than dextrose was also noted later by Deuel ('27) and more recently by Carpenter ('40). Higgins suggested that part of the increase of the respiratory quotient following ingestion of sucrose, especially when the quotient exceeds 1.0, is due to formation of fat. Lactic acid formation from the fructose fraction, with resultant liberation of carbon dioxide, has been suggested as a factor (Cathcart and Markowitz, '27; Campbell and Maltby, '28; Wierzuckowski and Laniewski, '31; Bachmann and Haldi, '37). Edwards, Bensley, Dill and Carpenter ('44), however, have clearly shown that after allowing for the effects of blood lactic acid, the corrected quotient was still greater after fructose than after the same amount of glucose. Regardless of the cause — combustion or synthesis — the relevant facts here are that the respiratory quotient increases rapidly; that it increases more rapidly than with dextrose, and that sucrose cannot produce such increase until the products of its hydrolysis have been absorbed from the alimentary tract.

Diabetics who require insulin and who suffer from hypoglycaemic reactions as the result of overdosage afford a unique opportunity for

testing the rapidity with which sucrose is hydrolyzed in the intestinal tract and the rapidity with which the product of this hydrolysis are absorbed by observation of (a) the response to sucrose ingestion subjectively and (b) the rapidity with which the ingested sucrose increases the concentration of sugar in the blood.

It is a well-known fact that, though the hypoglycaemia produced by an overdose of insulin may continue for hours in severe cases and require large amounts (hundreds of grams) of sugar to combat it, even when the latter is administered intravenously, in very mild cases the disagreeable symptoms may disappear within 2 or 3 minutes — in some cases in 1 minute — following ingestion of a little orange juice, provided the latter is taken on an empty stomach. For this reason, it has been a routine practice in the wards of The Montreal General Hospital since insulin was first used to treat all mild reactions in this manner. Oranges, however, in their natural state, contain approximately equal quantities of sucrose and dextrose (Roberts and Gaddum, '37). No conclusions, therefore, can be drawn from these experiences with respect to the rapidity with which sucrose increases the blood sugar. The experiences with diabetics in their homes, however, are of some significance. It has been the routine during the same period to advise such patients to carry with them at all times the ordinary commercial cubes of cane sugar, and the results have been equally satisfactory; dextrose has never been used in this clinic for the control of insulin reactions, except in very severe cases which required sugar intravenously Subjective improvement, however, is not as reliable as actual determination of the sugar content of the blood as an indication of the rapidity with which sugars or their derivatives are absorbed from the alimentary tract The purpose here, therefore, is to record the results of blood sugar determinations made in cases in insulin hypoglycaemia at 1-minute intervals following ingestion of sucrose.

METHOD OF INVESTIGATION

Selection of subjects

With large excesses of insulin, no increase of blood sugar may be noted for many hours even after administration of large amounts (hundreds of grams) of sugar (Rabinowitch, Fowler and Bensley, '37). Such cases, therefore, afford no indication of the rapidity with which the administered sugar is absorbed from the alimentary tract. The subjects used in this investigation, therefore, included only those who had very mild reactions.

Condition of subjects

Failure to note a rapid increase of blood sugar following ingestion of sugar may be due not to slow absorption directly but to a slow emptying of the stomach; it is well known, that food leaves the stomach most rapidly when it is administered on an empty stomach. In order, therefore, to exclude as much as possible artificially delayed absorption due to this factor, the only subjects studied were those who had insulin reactions in the fasting state, that is, in the morning, approximately, 15 hours after the last (evening) meal. Without exception, all of the subjects were being treated with protamine zinc insulin which, because of its prolonged action, tends to produce low blood sugars the following morning in the fasting state — 24 hours after its injection.

Sucrose drink

Aside from the degree of emptiness of the stomach, other conditions which effect the emptying time of the stomach are (a) the fluidity of the meal and (b) its osmotic pressure: the more liquid the meal, the sooner does it leave the stomach; hypotonic solutions leave the stomach more rapidly than isotonic solutions. In order, therefore, to avoid artificially delayed absorption of the ingested sugar due to these factors, the sucrose was administered in aqueous solution, and the latter was made hypotonic (5%).

Though the amount of sugar influences the time during which an increase of blood sugar may be prolonged, the height of the response is not proportional to the amount of sugar administered (MacLean and de Wesselow, '20–'21; Hansen, '23; Tisdall, Drake and Brown, '25). All subjects were, therefore, given the same amount, namely 10 gm.

Venous vs arterial blood sugars

In a normal person, after ingestion of a sugar, the arterial and venous blood sugars are equally affected until the blood sugar reaches a level of about 0.120%. Thereafter, for a time, the arterial blood sugar rises to a higher level than the venous (Foster, '23; Rabinowitch, '27). Therefore, since a normal person might have a blood sugar close to 0.120% before ingestion of sugar, analysis of the venous blood may fail to detect the beginning of absorption of the ingested sugar. Determination of true arterial blood sugar, however, involves radial artery or other arterial puncture which, at 1-minute intervals, is not practical. In cases of hypoglycaemia, however, where the blood sugars are well below 0.120% venous blood is as satisfactory as arterial. All tests were, therefore, made on venous blood.

Routine procedure

In each case, the concentration of sugar in the blood was determined before and then at 1-minute intervals after ingestion of the sucrose drink until the patient stated he "felt better".

As stated, with sufficiently large excesses of insulin, no change whatever may be noted in the blood sugar for many hours even in spite of absorption of hundreds of grams of sugar. The only subjects of interest here, therefore, were those in whom subjective improvement was noted very soon after the ingestion of the sucrose drink.

In the interpretation of the data, evidence of absorption of sugar was not regarded as positive unless the increase of blood sugar was in excess of 10 mg. per 100 ml. which is well in excess of the experimental error of the test used for the determination of the blood sugar. Blood sugar was determined by the Myers and Bailey ('16) modification of the Lewis-Benedict macro method, with all reagents crystallized repeatedly and the color determined by a colorimeter or by a photo-electric cell. The combined data of ten experiments (eight subjects) are shown in table 1.

DISCUSSION OF RESULTS

With three exceptions (experiments no. 2, 5 and 6), it will be noted that all bloods were markedly hypoglycaemic before the ingestion of the sucrose. The cases were, therefore, properly selected with regard to the reliability of venous blood sugars as an indication of the beginning of absorption of sugar from the alimentary tract.

No relationship was noted between the degree of hypoglycaemia and subjective disturbances — all reactions were mild — fitting in with previously reported experiences (Peters and Rabinowitch, '29; Rabinowitch, Fowler and Corcoran, '36) and now a well recognized phenomenon.

In all of the cases, subjective improvement was noted within 5 minutes of the ingestion of the sucrose, and it will be noted that of the ten experiments a definite increase of blood sugar (that is, in excess of 10 mg. per 100 ml. of blood) was noted within 1 minute in two cases, within 2 minutes in two cases, within 3 minutes in three cases, within 4 minutes in two cases and within 5 minutes in the remaining case.

A possibility which had to be considered was "emotional" hyperglycaemia due to discomfort from the needle punctures, that is, an increase of blood sugar not due to absorption of sugar from the alimentary canal, but to mobilization of sugar from its stores in the body. Though such hyperglycaemia undoubtedly occurs with marked pain

and fear (Bohm and Hoffmann, 1878; Cannon, Shohl and Wright, '11; Griffith, '23; Britton, '28) the writer, from experiences with thousands of blood sugar time curves, has never been impressed with the emotional effects of single needle punctures, provided the needle was sharp and each sample of blood was collected by an experienced person requiring one needle puncture only. In this study, however, unlike the ordinary blood sugar time curves for which blood samples are taken at ½- and 1-hour intervals, blood samples were collected at 1-minute intervals. To exclude "emotional" hyperglycaemia as much as possible, therefore, particular care was taken with regard to the sharpness of the needles

TABLE 1

Short period blood sugar time curves following oral administration of sucrose.

NO.	BLOOD SUGAR					
	Fasting state	Minutes after ingestion of sucrose				
		1	2	3	4	5
	%	%	%	%	%	%
1	0.056	0.050	0.066	0.103	0.133	
2	0.068	0.066	0.065	0.083	0.125	
3	0.063	0.060	0.064	0.065	0.063	0.111
4	0.045	0.044	0.084	0.093	0.129	
5	0.066	0.064	0.067	0.070	0.080	0.122
6	0.069	0.083	0.120			
7	0.040	0.073	0.083	0.125		
8	0.053	0.051	0.066	0.073	0.080	0.123
9	0.058	0.061	0.055	0.082	0.099	
10	0.045	0.046	0.046	0.053	0.068	0.100
A.M.	0.056	0.060	0.072	0.083	0.097	0.114

Note: No. 4 and 7 same subject 3 days apart. No. 6 and 8 same subject 11 days apart.

and all samples of blood were collected by the writer. As a control, however, in three experiments (nos. 8, 9 and 10) samples of blood were collected at 1-minute intervals for 5 minutes before the administration of the sucrose drink. The blood sugar data so obtained are shown in table 2, from which it will be noted that emotional hyperglycaemia was not a factor in the production of the increases of blood sugar noted soon after the ingestion of the sucrose drink. Fluctuations of the blood sugar were noted, but, without exception, all were slight and within the range of the experimental error of the test for the determination of blood sugar, i.e., less than 10 mg. per 100 ml. of blood.

The rapid increases of blood sugar noted after ingestion of sucrose in these experiments fit in with the above-mentioned rapid increase of

the respiratory quotient. The data for both the respiratory quotients and blood sugar indicate clearly that sucrose is very rapidly hydrolyzed and that the products of its hydrolysis are very rapidly absorbed. The impression, therefore, that dextrose is superior to sucrose as an immediate source of energy because it requires no preliminary digestion is without foundation. In fact, there is reason, experimentally and theoretically, to believe that sucrose, because of its laevulose fraction, is the superior sugar. Its apparently superior protein-sparing and ketolytic actions (Deuel, Gulick and Butts, '32) and its greater ability to maintain the blood sugar concentration during exercise (Dische and

TABLE 2

Short period blood sugar time curves in the absence of sugar administration.

SUBJECTS IN TABLE 1	BLOOD SUGAR					
	Before venous puncture	After venous puncture (minutes)				
		1	2	3	4	5
	%	%	%	%	%	%
8	0.051	0.049	0.056	0.050	0.054	0.053
9	0.053	0.059	0.056	0.061	0.060	0.058
10	0.040	0.046	0.045	0.044	0.049	0.045
A.M.	0.048	0.051	0.052	0.052	0.054	0.052

Goldhammer, '32) are examples. Whatever the mechanism may be, the fact of practical importance, and clearly demonstrated here, is that, though sucrose must be hydrolyzed before it is available as a source of energy, the hydrolysis is almost "explosive" in character and thus does not delay the availability of the sugar.

SUMMARY

Blood sugar time curves were obtained at 1-minute intervals following ingestion of sucrose, in order to determine the rapidity with which this food is available as a source of energy, using the increase of blood sugar as the indication of absorption from the alimentary tract.

Of the ten experiments reported here, a definite increase of blood sugar was noted within 1 minute in two cases, within 2 minutes in two cases, within 3 minutes in three cases, within 4 minutes in two cases and within 5 minutes in the remaining case. "Emotional" hyperglycaemia was excluded as a possible cause of the increase of sugar noted in three experiments.

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NEUROPATHOLOGIC STUDIES OF PANTOTHENIC ACID, BIOTIN AND FOLIC ACID COMPLEX DEFICIENCIES IN THE CHICK[1]

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The paralysis and neuropathologic lesions in pantothenic acid deficiency in the chick have been described by Phillips and Engel ('39). This deficiency was produced on the heated ration of Mickelsen, Waisman and Elvehjem ('38) who have described the dermatitis which occurred. The former authors found that in chicks fed the heated ration, there was a severe degeneration of the myelin and axons of the spinal cord and occasionally of the peripheral nerves. They found that pantothenic acid was necessary for the prevention of the neuropathologic lesions in the spinal cord and that riboflavin was necessary to prevent those in the peripheral nerves. Since that time, it has been shown by Waisman, Mills and Elvehjem ('42) that the heated ration was also deficient in other factors required in the nutrition of the chick. Due to these complicating factors, it seemed wise to reinvestigate the pathology of pantothenic acid deficiency in the chick as produced on the heated ration and on a complete sucrose ration.

EXPERIMENTAL

The heated ration used was the modification of the heated diet used by Waisman, Mills and Elvehjem ('42). It had the following percentage composition: ground yellow corn, 56; standard wheat middlings, 25; crude casein, 12; soybean oil, 3; and a salt mixture, 4. The chicks were given 2 drops of halibut liver oil weekly. Although the original workers heated the grain and casein portion of the ration for 100 hours at 100°C. to destroy pantothenic acid, later work showed that

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a 30-hour treatment of the ration (Mickelsen, Waisman, and Elvehjem, '38) at 120°C. gave a more consistent incidence of dermatitis and paralysis.

In this series of experiments each 100 gm. of the basal ration was supplemented with 300 μg. of thiamine, 400 μg. of riboflavin, 400 μg. of pyridoxine, 100 mg. of choline, and 0.50 mg. of 2-methyl, -1, 4-napthoquinone. There was no growth stimulation when these were added to the basal ration, but the chicks did survive for a longer period.

Since the rate of growth was very far below the maximum even when an ample amount of pantothenic acid was added to the basal ration, various supplements were added to improve the growth and to study the effect on the accompanying pathologic lesions. Incidentally, it was possible to study the pathologic changes in a mild type of biotin deficiency which developed when 10% cartilage and 3% solubilized liver fraction were added to the basal heated ration supplemented with 15 mg. of calcium pantothenate per kilogram.

In order to study the neuropathologic lesions of pantothenic acid deficiency, uncomplicated by other factors, in contrast to the chicks fed the supplemented heated ration, a sucrose ration was used. Its percentage composition was as follows: sucrose, 54; kidney residue, 3; cartilage, 15; alcohol-extracted casein, 18; salts IV [2] 5, and soybean oil 5, and a factor concentrate equivalent to 10% of yeast. One hundred gm. of the basal ration were supplemented with 300 μg. of thiamine, 400 μg. of riboflavin, 400 μg. of pyridoxine, 100 mg. of choline, and 10 mg. of nicotinic acid. The chicks were given 2 drops of halibut liver oil weekly. Supplements of calcium pantothenate of from 2 to 20 mg. per kilogram were added to study the rate of alleviation of the pantothenic acid deficiency.

In order to eliminate the possibility of a deficiency of the folic acid complex being involved in the pantothenic acid deficiency produced on either the heated or the sucrose basal rations, deficiency of the folic acid complex was produced by the use of the following ration: dextrin, 56.7%, alcohol extracted casein 18%, salts IV 5%, $CaHPO_4$ 1%, soybean oil 5%, liver residue 4%, gelatin 10%, and cystine 0.3%. This ration was supplemented with thiamine, riboflavin, pyridoxine, choline, nicotinic acid, and halibut liver oil in the same amounts as in the basal ration itemized in the preceding paragraph.

The deficient chicks were autopsied when they became so severely deficient that death was imminent. The corresponding controls were simultaneously sacrificed for comparison.

[2] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935).

The sciatic nerves were studied by means of the polarizing microscope (Setterfield and Sutton, '35), the Marchi osmic acid reaction (Swank and Davenport, '34) and by the silver impregnation method of Bodian ('36). The spinal cords were studied after they were stained by Einarson's method ('32), by the Marchi reaction and by the silver impregnation method of Bodian ('36).

RESULTS

On the heated ration only, there was a severe myelin degeneration in the spinal cord in all four chicks. When 15 mg. of calcium pantothenate were added to the basal ration, no paralysis was observed and there were no pathologic lesions in the spinal cord of the six chicks. When a solubilized liver fraction supplement at a 3% level was added as a source of the folic acid complex, there was a very appreciable increase in the rate of growth. When a supplement of 10% cartilage or a supplement of 3% glycine, 0.5% arginine and 0.5% cystine was added as a source of the cartilage factor, there was a further increase in growth. In both of these latter groups, a very pronounced biotin deficiency was present as evidenced by the presence of a dry scaly callous condition on the bottom of the feet and fissuring between the toes (Hegsted, Oleson, Mills, Elvehjem and Hart, '40, and Hegsted, Mills, Briggs, Elvehjem and Hart, '42). It was found that there was no evidence of any lesion in the spinal cord or in the sciatic nerves of these chicks that were mildly deficient in biotin.

On the sucrose ration which was complete in all known factors except for pantothenic acid, dermatitis and paralysis were observed in all six chicks. The same type of myelin and axon degeneration was found in the spinal cord as was present in the chicks which developed paralysis on the heated ration. When 2 mg. of calcium pantothenate were added per kilogram of ration, there was still a severe degree of myelin degeneration in the spinal cords of the four chicks in the group. When 3 mg. of calcium pantothenate were added per kilogram, the lesions in the spinal cord were still quite pronounced in all four chicks. However, a supplement of 5 mg. of calcium pantothenate per kilogram prevented all but a slight trace of the myelin degeneration. Levels of 10 and 20 mg. of calcium pantothenate per kilogram completely prevented the neuropathologic lesions. There was no case in which any myelin degeneration was observed in the sciatic nerve.

The lesions in the spinal cord were characterized by a widespread myelin degeneration in all areas of the white matter. This occurred to the greatest extent in the lateral and anterior columns and extended

from the cervical region to the lumbar region of the cord. The axons in these regions frequently showed mild degenerative changes although not to the same extent as the accompanying myelin degeneration. Some very mild changes were observed in the Nissl materials of the nerve cells in the cervical and brachial regions of the spinal cord.

The chicks on the ration deficient in the folic acid complex did not have any of the incrustations about the beak and feet that occurred in pantothenic acid deficiency nor the callousness and fissuring of the feet that occurred in biotin deficiency. The chicks were not too well feathered. After 3 or 4 weeks on the ration they became so weak as to be unable to stand. They would rest on the floor of the cage and when they were disturbed, they seemed to be unable to get up on their feet. They appeared to have a good sense of balance but were too weak to support their own weight.

No lesions were found in the spinal cord of the seven deficient chicks. In two of these chicks there was a very mild myelin degeneration in the sciatic nerve apparently unaccompanied by any axon change. It was found impossible to keep these chicks alive once they became so weak that they could not support their own weight. It was thus impossible to determine if these occasional lesions in the sciatic nerve would become more severe in a prolonged deficiency. No lesions were found in the nerve tissues or the control groups which received 2% solubilized liver fraction or a folic acid concentrate equivalent to 5% solubilized liver fraction.

DISCUSSION

The neuropathologic lesions of pantothenic acid deficiency were studied in chicks on the heated ration supplemented with the crystalline vitamin B complex vitamins and on a sucrose ration which was believed to be otherwise complete. The pathological changes in the spinal cord, which were reported previously by Phillips and Engel ('39) in chicks on the unsupplemented heated ration and which were prevented by pantothenic acid, were seen on both of these improved rations. The deficiencies which were still present in the supplemented heated ration did not complicate the picture of the spinal cord lesions in the pantothenic acid deficient chicks. On the heated ration, the primary and most severe deficiency was that of pantothenic acid. Not until sufficient pantothenic acid was supplied did the effect of biotin and folic acid complex deficiencies appear. A study of the mild biotin deficiency and of an acute deficiency of the folic acid complex revealed no neuropathologic changes. Therefore, it was concluded that the myelin and axon

degeneration observed in the spinal cord in pantothenic acid deficiency in the chick was due to the deficiency of pantothenic acid alone and not to the lack of any other nutritional factor.

The fact that myelin and axon degeneration occurred in the spinal cord and not in the peripheral nerves of the pantothenic acid deficient chick is unexplained. In both thiamine and riboflavin deficiencies, in chicks, the first degeneration of myelin to be observed was in the peripheral nerves and not in the spinal cord. If the spinal cord becomes involved at all in these cases it is only much later in the progress of the deficiency. However, in pantothenic acid deficiency the spinal cord alone was involved. The areas of the spinal cord which were involved in pantothenic acid deficiency were the lateral and anterior columns. These were usually free of any degeneration in a riboflavin deficiency.

SUMMARY

1. The deficiency of pantothenic acid in the chick caused a very widespread myelin and axon degeneration in the spinal cord. There was no accompanying degeneration in the peripheral nerves.

2. These lesions of the spinal cord occurred in pantothenic acid deficiency, whether it was produced on a very incomplete ration such as the heated ration or on an otherwise complete sucrose ration.

3. Complicating deficiencies which appeared on the heated ration did not seem to alter the neuropathologic lesions in a pantothenic acid deficiency.

4. No neuropathologic lesions were observed in chicks suffering from a mild biotin deficiency, or an acute deficiency of the folic acid complex.

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NEUROPATHOLOGIC STUDIES OF ACUTE AND CHRONIC THIAMINE DEFICIENCIES AND OF INANITION [1]

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TWO TEXT FIGURES AND ONE PLATE (NINE FIGURES)

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Prickett, Salmon and Schrader ('39) observed differences between the pathological changes in acute and chronic thiamine deficiencies. Rats with acute thiamine deficiency had only a few fibers that showed Wallerian degeneration. However, nerves of deficient rats, that became moribund without any evidence of the neuromuscular symptoms which are typical of the acute stage of the deficiency, showed a much more extensive myelin and axon degeneration. Zimmerman ('39, '40) emphasized the difference between the acute and chronic states of thiamine deficiency in the pigeon, rat and dog. Street, Zimmerman, Cowgill, Hoff and Fox ('41) further discussed the effects of a long-continued subminimal intake of thiamine. In dogs, they observed a moderate spasticity of the hind legs, unsteadiness, staggering and vomiting, with the absence of the deep reflexes of the hind legs. Histological studies of the nervous system revealed an extensive myelin degeneration of both the peripheral nerves and the posterior columns of the spinal cord. These lesions were observed on a diet which contained 8% autoclaved yeast. Swank ('40) also reported appreciable differences in the neuropathology of pigeons in acute and chronic thiamine deficiencies.

Several authors have discussed the effect that the inanition, which always occurs early in the thiamine deficiency, has on the pathology observed. The need for consideration of this complicating factor is a real one, especially in those cases where a prolonged chronic deficiency is produced. Prickett, Salmon and Schrader ('39) and Street, Zimmerman, Cowgill, Hoff and Fox ('41) reported myelin degeneration in the

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peripheral nervous system of the pigeons, dogs and rats which had received a restricted intake of a ration containing ample thiamine. By this means they simulated a starved condition similar to that developed by chronically thiamine deficient animals. These authors stated that the changes were less severe and less frequent in starved, than in chronically thiamine deficient animals.

Swank ('40) reported that the blackening of myelin sheaths by the Marchi method was not pathognomic of degeneration. He stated that the blackening which occurred in the peripheral nerves of starved pigeons was not truly a myelin degeneration.

Due to the great confusion in the literature which has arisen because of the different degrees of thiamine deficiency studied by various workers and the complicating factor of inanition, it was decided to study the pathology of inanition and of acute and chronic thiamine deficiencies, as nearly as possible uncomplicated by any other nutritional deficiency.

EXPERIMENTAL

The first experiment was done with 6- to 8-week-old pigeons in a manner similar to that described by Swank ('40). Preliminary trials were made with Swank's diet II the percentage composition of which was corn starch 66, alcohol-extracted casein 20, cod liver oil 4, peanut oil 6, and salts IV [2] 4. This ration was supplemented with a vitamin K concentrate [3] at a level equivalent to 3 Almquist units per pigeon per day. After preliminary trials, this ration was changed considerably, in order (1) to make it more suitable for feeding by pipette, (2) to improve its digestibility, thus reducing weight loss, and vomiting, but increasing the survival time, and (3) to reduce the fat level in order to make the ration less thiamine-sparing. The corn starch was replaced by dextrin, the peanut oil was reduced from 6 to 4%, and the cod liver oil left out of the ration. The basal ration then consisted of: dextrin 72%, casein 20%, peanut oil 4%, and salts IV 4%. It was supplemented with adequate irradiated ergosterol, shark liver oil, and the vitamin K concentrate.

This ration was mixed with enough distilled water to make it sufficiently fluid to allow it to be pipetted through a 50 ml. quick-delivery pipette. This suspension of the ration was made daily. The pigeon was held by a strip of Gooch rubber tubing which was snugly wrapped around its breast and wings and fastened with a hemostat. By this means, it was possible to immobilize the bird without interfering with

[2] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935).
[3] Abbott's Klotogen.

its normal breathing, until a known amount of ration suspension could be introduced into its crop.

The pigeons of the first group were fed only 4 gm. of the basal ration daily, in order that they would be starved as well as rendered acutely deficient in thiamine. Those in the second group were fed 10 to 15 gm. of the basal ration per day, enough to prevent complication of the acute deficiency by starvation effects. Those in the third group were fed 10 to 15 gm. of the basal ration but were given a minimal daily injection of thiamine intramuscularly to produce a chronic thiamine deficiency, supposedly uncomplicated by any starvation effects. Those in the fourth group were given 4 gm. of the basal ration plus a daily injection of 100 μg. of thiamine, in order that they would be starved but not deficient of the vitamin. The pigeons in the fifth group were given ample ration plus 100 μg. of thiamine daily.

Each of the five groups was subdivided into three subgroups. The pigeons in subgroup A of each group were given the basal ration. Those in subgroup B were given the basal ration, supplemented with 100 μg. of riboflavin and 0.5 mg. of pantothenic acid per day. Those in subgroup C received the basal ration, supplemented daily with a gelatin capsule containing 100 μg. of riboflavin, 100 μg. of pyridoxine, 0.5 mg. of pantothenic acid, 15 mg. of choline, 15 mg. of inositol, 1 mg. of nicotinic acid, 15 mg. of para-aminobenzoic acid and 0.5 gm. of a sulfite treated 1:20 liver extract.

Day-old chicks were used in the second experiment. They were found to be more satisfactory animals, since a complete ration was available which could be made stringently deficient in thiamine by the omission of crystalline thiamine. Ten groups of chicks were used as outlined below.

The basal ration used in this experiment consisted of: dextrin 54.7%, alcohol-extracted crude casein 18%, gelatin 10%, salts IV 5%, $CaHPO_4$ 1%, soy bean oil 5%, liver residue 4%, solubilized liver extract 2%, and cystine 0.3%. This mixture was supplemented with crystalline vitamins at the following levels per kilogram: riboflavin 6 mg., pyridoxine 4 mg., pantothenic acid 15 mg., choline 1.5 gm., and nicotinic acid 100 mg. Two drops of halibut liver oil were given weekly to each chick. This ration, when supplemented with 4 mg. of crystalline thiamine per kilogram, gave good growth in chicks. Since it was impossible to feed day-old chicks by pipette, it was necessary to allow them to consume the ration ad libitum.

The chicks in group 1 were fed the basal ration to produce an immediate, acute thiamine deficiency. Those in group 2 were first given

the basal ration plus a 20 μg. injection of thiamine daily for 2 weeks. Then an acute thiamine deficiency was allowed to develop.

The chicks in group 3 were fed the basal ration until symptoms of an acute deficiency began to appear. Then subminimal injections of thiamine were given daily in order to prevent death and to alleviate partially the opisthotonus of the acute deficiency. The injections were continued until all signs of the acute deficiency had disappeared and the chicks had become chronically paralyzed. Those in group 4 were given the basal ration plus 20 μg. of thiamine daily for 2 weeks only. When the acute symptoms began to appear, subminimal injections of thiamine were given as in group 3. In groups 5, 6, 7, and 8, the chicks were treated like those in group 4 except that the following daily injections were given: 20 μg. of riboflavin to those in group 5, 100 μg. of pantothenic acid to those in group 6, 20 μg. of pyridoxine to those in group 7, and 20 μg. of riboflavin, 100 μg. of pantothenic acid, and 20 μg. of pyridoxine to those in group 8.

The chicks in group 9 were fed the basal ration plus an injection of 40 μg. of thiamine per day. Their daily intake was limited to that of individual chicks in the chronic thiamine deficient groups. Those in group 10 were allowed to consume the basal ration ad libitum and were given a thiamine injection of 40 μg. per day.

In each of these experiments, when the deficient or starved pigeons and chicks became so severely affected that death was imminent, they were killed and autopsied. Controls, which had received ample thiamine were sacrificed and autopsied in the same manner. The brain, the brachial segment of the spinal cord, one brachial and sciatic nerve were fixed in a solution consisting of: 95% ethanol 100 parts, glacial acetic acid 5 parts, and paraldehyde 2 parts. After dehydration, imbedding and sectioning, these tissues were stained by the Bodian silver technique ('36). A cervical portion of the spinal cord, one brachial, one sciatic and the nerve to the pectoralis muscle were fixed in a 10% formalin solution buffered at a pH of 7.0. The spinal cord and one-half of the brachial and sciatic nerves and the nerve to the pectoralis muscle were stained, by a modified Marchi osmic acid method (Swank and Davenport, '34). The other half of the brachial and sciatic nerves were used for polarizing microscope studies of the birefringent materials between crossed Nicols (Setterfield and Sutton, '35).

In order to study the pyruvic acid metabolism of these thiamine deficient chicks, pyruvic acid analyses were made of the blood, sciatic nerve and gastrocnemius muscle. The method used for these analyses was that of Bueding and Wortis ('40).

RESULTS

Pigeons

The observations of the pigeons are reported in table 1. In group 1 (acutely thiamine deficient and starved) there was a mild myelin and axon degeneration in the sciatic and brachial nerves of six of the ten pigeons and a mild myelin and axon degeneration in the spinal cord of one of them. A daily supplement of all the known crystalline members of the vitamin B complex did not appear to alter the combined effects of the acute thiamine deficiency and the starvation.

In group 2 (acutely thiamine deficient but not starved), a mild myelin degeneration was observed in the peripheral nerves of six of the eleven pigeons. There was also a mild myelin degeneration of the spinal cord in four of them. However, these changes were of a minor nature and there was only slight axon degeneration in the peripheral nerves and the spinal cord. The vitamin supplements did not prevent any of the lesions.

In group 3 (chronically thiamine deficient but not starved), a very definite myelin and axon degeneration was observed in the peripheral nerves of thirty-seven of the thirty-eight pigeons. There was some degeneration in the spinal cord (fig. 11) but this was neither as frequent nor as severe as that observed in the peripheral nerves (figs. 8 and 10). The degenerated fibres in the spinal cord were not localized in any definite region. The posterior columns, however, were almost completely free of any degenerative changes. The leg weakness that developed in these pigeons was never comparable in extent to that seen in the corresponding chicks in experiment 2 which is described later.

The chronically thiamine deficient pigeons did not readily digest the ration which was introduced into their crops. As they became more chronically deficient, there was a gradual reduction in the amount of food which they could pass into the intestinal tract from the crop. Atony of the crop appeared to develop and the purpose of forced feeding was in this way defeated. The digestive system of these pigeons seemed capable of handling only a very small amount of ration, approximately comparable to that actually eaten by a pigeon that is chronically deficient.

In group 4 (starved but ample thiamine), there was observed a mild myelin degeneration of the peripheral nerves and spinal cord comparable to that observed in group 1. The vitamin supplements and sulfited 1:20 liver extract seemed to have no preventative effect on the development of the lesions.

TABLE 1

Birds which developed histopathological changes in the spinal cord and sciatic nerve.

GROUP NO.	SUB-GROUP	RATION	TYPE OF DEFICIENCY	NO. OF BIRDS	NO. OF BIRDS SURVIVING	SPINAL CORD	SCIATIC NERVE
		Experiment 1 — Pigeons					
1	A	Basal only — restricted intake	Acute and starved	4	4	1	2
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	Acute and starved	4	3	0	2
	C	A + vitamin B complex mixture	Acute and starved	4	3	0	2
2	A	Basal only — ample	Acute	4	4	1	2
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	Acute	4	4	1	2
	C	A + vitamin B complex mixture	Acute	4	3	2	2
3	A	Basal — ample + subminimal thiamine	Chronic	12	11	3	11
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	Chronic	20	19	9	18
	C	A + vitamin B complex mixture	Chronic	12	8	5	8
4	A	Basal + 100 μg. B_1/day — restricted intake	Starved	8	8	3	4
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	Starved	12	9	2	2
	C	A + vitamin B complex mixture	Starved	8	5	0	2
5	A	Basal + 100 μg. B_1/day — ample ration	None	4	3	0	1
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	None	4	3	0	1
	C	A + vitamin B complex mixture	None	4	4	1	1
		Experiment 2 — Chicks					
1		Basal only	Acute	5	5	0	0
2		Basal + 20 μg. B_1/day for 2 weeks — then basal only	Acute	14	14	1	2
3		Basal + subminimal injection of B_1 daily	Chronic	10	5	3	5
4		Basal + 20 μg. B_1/day for 2 weeks — then subminimal injection of B_1 daily	Chronic	30	26	8	25
5		4 + 20 μg. riboflavin/day	Chronic	17	15	6	14
6		4 + 100 μg. pantothenic acid/day	Chronic	17	13	5	12
7		4 + 20 μg. pyridoxine/day	Chronic	17	15	5	15
8		4 + 20 μg. riboflavin + 100 μg. pantothenic acid + 20 μg. pyridoxine/day	Chronic	17	12	4	11
9		Basal + 40 μg. B_1/day restricted intake	Starvation	13	13	5	13
10		Basal + 40 μg. B_1/day ad libitum	None	14	14	0	0

The pigeons of group 5 (ample thiamine and ample ration) showed no pathological lesions (fig. 3) except in a few isolated cases. The ration was still so deficient that no growth was observed although those in subgroup C did maintain their original weight.

Chicks

The results observed in the chicks in experiment 2 are recorded in table 1. In the chicks of group 1 subjected to an immediate, acute thiamine deficiency, the first acute deficiency signs appeared after 8 or 9 days. Typical head retractions had developed by that time and the chicks were dead within 24 hours after the onset of these symptoms. No definite pathology could be seen in either the sciatic nerve (figs. 5 and 6) or the spinal cord. In group 2 where an acute deficiency was produced after 2 weeks of normal growth, opisthotonus appeared 7 to 10 days after the daily injections of thiamine had been discontinued. Again, the acutely deficient chicks died within 24 hours after the onset of the visible symptoms. In two of the fourteen chicks autopsied, a slight speckling was observed in the myelin of the sciatic nerve and of the spinal cord; the remainder appeared to be completely normal. The changes observed in these two chicks were not typical of any described stage of myelin degeneration.

In group 3, a chronic thiamine deficiency was produced in only five of the ten chicks; the others died in a more acute stage. Due to the sensitivity of the young chick to thiamine deficiency, it was very difficult to keep them alive once the symptoms of acute thiamine deficiency became evident. In each case the chronic deficiency was not a prolonged one. Five of these chronically deficient chicks showed a mild myelin degeneration in the sciatic nerve; and three of these had a very mild degeneration in scattered areas of the spinal cord.

When the chicks were allowed to grow normally for 2 weeks, as in groups 4–8 inclusive, it was possible to produce a chronic thiamine deficiency more readily. This was done by the injection of a subminimal dose of 2–4 μg. of thiamine daily. In these chicks which weighed an average of 110 gm. at the appearance of deficiency symptoms, these symptoms could be alleviated by this small daily injection. However, enough thiamine to permit a complete recovery was never given. Thus, the chicks could be maintained in a state of chronic thiamine deficiency for as long as 3 weeks. A very noticeable and severe leg paralysis developed after 4–7 days in this chronic state. In the early stages of the paralysis (fig. 1), the toes were curled inwards slightly. As the chronic

condition was prolonged, the "curled toe" condition was replaced by a rigid extension of the legs (fig. 2). In almost every case in groups 4–8 where a chronic thiamine deficiency was produced, there was a very definite myelin degeneration in the sciatic nerve (fig. 9) followed by a comparable axon degeneration. Thirty % of these chicks had a mild, scattered myelin degeneration in the spinal cord. When supplements of riboflavin, pantothenic acid, and pyridoxine were given singly or collectively, there was no alleviation of the gross or pathological changes.

Fig. 1 A chick in an early stage of the paralysis observed in chronic thiamine deficiency.
Fig. 2 A chick in a much later stage of the same type of paralysis.

The chicks in group 9 which received a restricted amount of the ration equivalent to that eaten by chicks of the same weight in group 4, showed no signs of "curled toe" or extension of the legs. Instead they became very weak and flaccid with no sign of any rigidity of the leg even in the final stages of starvation. A rather unexpected observation was noted in connection with these starved chicks because they died slightly sooner than those with a chronic thiamine deficiency. There was a mild myelin degeneration in the sciatic nerves (fig. 7) and spinal cord of all the starved chicks. This degeneration seemed to be accompanied by some slight changes in the axon cylinders.

The chicks in group 10 which received the ration ad libitum with ample thiamine, grew normally and showed no signs of paralysis. Upon autopsy and histological examination, the spinal cord and the sciatic nerve (fig. 4) appeared to be normal in all respects.

The pyruvic acid concentration in the blood of six chronically thiamine deficient chicks averaged 6.3 mg. per 100 ml. in comparison with an average of 2.3 mg. per 100 ml. in six chicks which received ample thiamine. The pyruvic acid concentration was 0.17 mg. per gram in the sciatic nerve of the deficient chicks and 0.09 mg. per gram in the controls. There was little difference in the levels in the gastrocnemius muscle.

DISCUSSION

The differences in the outward symptoms and the pathological lesions which we observed in the acute and chronic phases of thiamine deficiency in the pigeon and the chick have been described above.

In pigeons with an acute thiamine deficiency but which received an ample amount of ration, a mild myelin degeneration was observed. However, in chicks with an uncomplicated, acute thiamine deficiency, no neuropathological lesions were observed. This difference may be accounted for by the much longer period necessary to produce an acute deficiency in the pigeon than in the young, growing chick. The complicating deficiencies that were present in the modified Swank pigeon ration may also be involved. The nutrition of the pigeon is not understood sufficiently well to permit one to evaluate the full extent of the inadequacy of this ration. However, it is significant that we found supplementing with the known crystalline B complex vitamins and sulfited 1:20 liver extract did not make the ration adequate.

It was observed that, in a prolonged thiamine deficiency in the pigeon and the chick, there was a definite myelin and axon degeneration of the peripheral nerves and in some cases, a similar degeneration in the spinal cord. The severity of the peripheral nerve lesions was greatest in the chicks in experiment 2 where the ration was complete in all known respects except thiamine. These observations were in accord with those of chronic thiamine deficiency made by Prickett, Salmon and Schrader ('39), Zimmerman ('39, '40), Street, Zimmerman, Cowgill, Hoff and Fox ('41), and Swank ('40).

Prolonged chronic thiamine deficiency leads to very definite complications because of the severe anorexia which thiamine deficient animals develop. Prickett, Salmon and Schrader ('39), and Street, Zimmerman, Cowgill, Hoff and Fox ('41) have reported a myelin degeneration in the peripheral nervous system of animals which had been maintained for long periods of time on the amount of food consumed by the thiamine deficient animals. These authors reported that the lesions observed in inanition were less severe than the ones observed in the prolonged thiamine deficient animals.

The work of Swank ('40); Swank and Bessey ('41), has not completely clarified the relation between the pathology of a prolonged thiamine deficiency and of inanition. He reported that in his forced-fed pigeons the lesions of the central nervous system observed in the chronically thiamine deficient birds were due to the thiamine deficiency and not to inanition. He believed that the daily introduction of a sufficient volume of ration into the crop of the pigeon, insured the digestion and utilization of the ration. However, our observations do not support this assumption. After the pigeons had been forced-fed for a period long enough to produce the initial symptoms of a mild chronic thiamine deficiency, the birds were observed to utilize only a small amount of the ration introduced daily into their crops. The pigeons were sufficiently accustomed to the forced feeding to endure this without vomiting. McCarrison ('19) postulated that a large part of the clinical and morbid anatomical effects observed in pigeons on an exclusive dietary of autoclaved rice could be attributed to a derangement of function of the organs of digestion and assimilation. This may explain the failure of the pigeon to utilize an adequate amount of food. The natural tendency of the bird to reduce its caloric intake during the thiamine deficiency could not be overcome by introducing food into the upper part of the digestive tract.

The role that inanition plays in the chronic thiamine deficiency cannot be too highly emphasized. Wolbach ('37), stated that a rational account of the pathological consequences of the deficiency of the B complex was not possible. He believed that it was better to regard the primary pathological effects of thiamine deficiency as not yet demonstrable and to regard the changes observed as secondary to the effects of inanition. Our observations support this view and these experiments with pigeons and chicks indicate that inanition is one of the principle factors involved. However, it should be pointed out that more severe degenerative changes occurred as the result of chronic thiamine deficiency than of inanition alone.

It is as yet impossible to explain why the control chicks which received the amount of ration voluntarily consumed by the chronically thiamine deficient chicks of the same weight died earlier than the deficient ones. The metabolism of the animal subjected to a prolonged period of partial thiamine deficiency is not understood. The role that inanition and other factors play in the production of neuropathological lesions during a chronic thiamine deficiency cannot be fully differentiated from the effects of the thiamine deficiency itself.

SUMMARY

1. There was a mild myelin degeneration in the peripheral nerves and in the spinal cord of the pigeons that had an acute thiamine deficiency. However, there was no evidence of any such degeneration in acute thiamine deficiency in chicks.

2. A mild myelin and axon degeneration was observed in the peripheral nerves and sometimes in the spinal cord of the pigeons and chicks which were maintained on a greatly restricted intake. These changes were more severe than those occurring in any acute deficiency produced in the pigeon.

3. Moderate degeneration occurred in the peripheral nerves and occasionally a mild degeneration in the spinal cord of the pigeons and chicks on a chronic thiamine deficiency. These changes were more severe than those occurring in inanition.

4. The pyruvic acid level of the blood of chronic thiamine deficient chicks was considerably higher than that of the controls. A slight increase in the pyruvic acid level of the sciatic nerve was detected but no change was observed in the gastrocnemius muscle.

These data do not warrant the conclusion that the neuropathology observed in a chronic thiamine deficiency can be attributed solely to a thiamine deficiency uncomplicated by other factors.

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PLATE 1

EXPLANATION OF FIGURES

3 A longitudinal section of the brachial nerve of a pigeon which received ample thiamine and the ration ad libitum. Normal myelination. Crossed Nicols. × 440.

4 A longitudinal section of the sciatic nerve of a chick which received ample thiamine and the ration ad libitum. Normal myelination. Marchi stain. × 440.

5 A longitudinal section of the sciatic nerve of a chick in a state of acute thiamine deficiency. Normal myelination. Crossed Nicols. × 440.

6 Another longitudinal section of the nerve in figure 5. Normal myelination. Marchi stain. × 440.

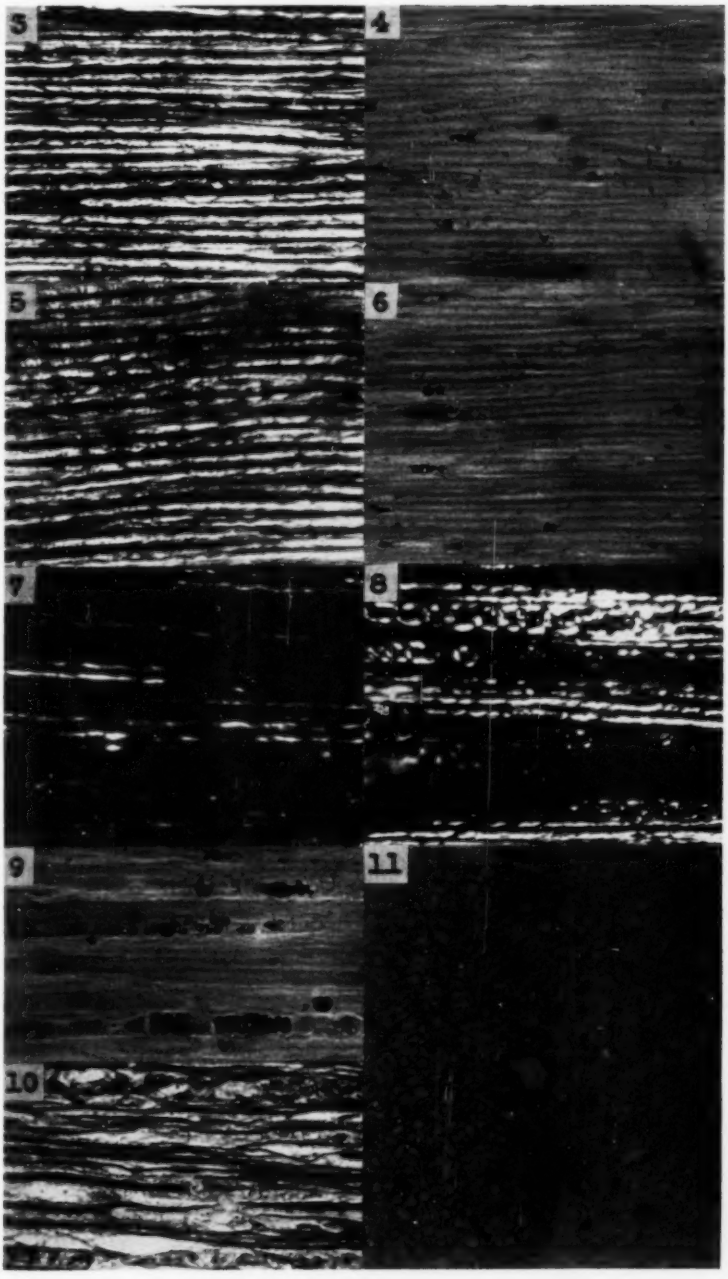
7 A longitudinal section of the sciatic nerve of a chick which had received the amount of ration consumed by a chronically thiamine deficient chick. Scattered myelin degeneration. Crossed Nicols. × 440.

8 A longitudinal section of the sciatic nerve of a pigeon in a state of chronic thiamine deficiency. Widespread myelin degeneration. Crossed Nicols. × 440.

9 A longitudinal section of the sciatic nerve of a chronically thiamine deficient chick. Widespread myelin degeneration. Marchi stain. × 440.

10 A longitudinal section of the sciatic nerve of a chronically thiamine deficient pigeon. Widespread axon degeneration. Bodian Ag stain. × 440.

11 A transverse section of the spinal cord of a chronically thiamine deficient pigeon. Scattered myelin degeneration. Marchi stain. × 440.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EFFECTS ON THE ALBINO MOUSE OF FEEDING DIETS VERY DEFICIENT IN EACH OF SEVERAL VITAMIN B FACTORS

(THIAMINE, RIBOFLAVIN, PYRIDOXINE AND PANTOTHENIC ACID)[1]

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ONE FIGURE

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As part of the investigations in this laboratory, on the relation of diet to resistance to infection with the virus of poliomyelitis, the response of mice to single deficiencies of four members of the vitamin B group has been studied in some detail. In particular, was it necessary to know how the separate deficiencies would make themselves manifest and how long the animals would live after each deficiency became evident. The rat has been used extensively in studies on the several vitamins B, but less information is available concerning the manner in which an inadequate supply of these dietary essentials affects the mouse. It has, however, been amply demonstrated that there is a considerable difference in the response of these two species to deficiencies of some of the B factors.

Mice on a diet markedly deficient in vitamin B_1 become hyperirritable and develop anorexia and atonia, which are followed by death, but do not show several of the symptoms seen in the rat and considered to be specific for vitamin B_1 deficiency, such as loss of proprioceptive sense and a tendency to move in circles (Freudenberg and Cerecedo, '31; Hauschildt, '42; Woolley and White, '43). Likewise Foy and Cerecedo ('41) have reported that pyridoxine is essential for the mouse but in its absence there is no dermatitis such as that frequently observed in the rat on certain types of pyridoxine-deficient diets. On the other hand, the only consistent sign of a lack of riboflavin in the rat,

[1] Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

besides a failure of growth, consists of changes in the eyes, and these may in some cases be discernible only upon special examination, whereas Lippincott and Morris ('41–'42) have described other riboflavin-deficiency symptoms in the mouse. The outstanding characteristics of these symptoms were loss of hair in certain areas, superficial cracks and fissures about the snout and on the front legs, exudate on the conjunctivae and about the mouth, a thickening and distortion of the ears, a hunched back while standing, and an abnormal gait with mild ataxia. Not all of these symptoms were observed in all of the animals, and a state of chronic deficiency produced more exaggerated symptoms than when the deficiency was acute.

Several investigators have studied pantothenic acid deficiency in the mouse (Morris and Lippincott, '41–'42; Sandza and Cerecedo, '41; Woolley, '41). The most commonly observed sign of pantothenic acid deficiency in the mouse appears to be alopecia, but Woolley has also reported hyperirritability, lack of muscular control followed by paralysis, and an abnormal condition of the eyes which, in some cases, could not be opened.

In the experiments reported below the vitamin B factors were supplied as pure compounds.[2] A deficiency of each of four factors, thiamine, riboflavin, pyridoxine, and pantothenic acid, was studied separately by omitting completely from the diet the factor under consideration. Results obtained in general confirm those reported in the literature but are more extensive, especially with respect to length of survival. They differ in certain other details.

EXPERIMENTAL

Two separate experiments were conducted, which, apart from some minor details, were similar except that the animals were raised on different stock diets. The mice used in the first experiment (experiment A) were raised on our regular stock diet which is partially composed of purified ingredients and has previously been described (Foster, Jones, Henle and Dorfman, '44). For the second experiment (experiment B) the animals were raised on a commercial dog food.[3] The two stock diets were used to determine if a difference in this respect would produce dissimilar results when the animals were put on the deficient diets. In each experiment the split-litter technique was used and the animals were distributed among five groups. There was considerable difference in the ages of the various animals, and at the time of start-

[2] These pure compounds were supplied by Merck and Company.

[3] Purina small dog checkers.

ing, their ages varying from 28 to 41 days in both experiments, with the age of about 80% of the mice falling between the 32nd and 38th days.

Group I was given a synthetic diet which was adequate for satisfactory growth; the composition of this diet is shown in table 1. The other groups of animals were given the same ration except that in each case one of the B factors was omitted, as follows: group II, thiamine; group III, riboflavin; group IV, pyridoxine, and group V, calcium pantothenate. In each experiment there were twenty animals in a group, with the exception of group I of experiment B, which contained nineteen animals. Experiment A was continued for 205 days, at which time all of the animals of groups II, III and IV, and all but four mice in group V, had died. Experiment B was continued for 135 days, at

TABLE 1

Dietary ingredients.

BASAL DIET		VITAMIN B MIXTURE	
	Parts		*mg./100 gm.*
Labco casein	25.0	Thiamine chloride	0.2
Cellulose	2.0	Riboflavin	0.5
Salt mixture[1]	4.0	Pyridoxine	0.2
Linseed oil	1.5	Calcium pantothenate	5.0
Wheat germ oil	1.0	Nicotinic acid	10.0
Fish liver oil concentrate	0.008	Inositol	10.0
Cerelose	63.5	P-aminobenzoic acid	10.0
	97.008	Choline chloride	30.0
Vitamin B mixture	3.0	*The above was carried on:*	
	100.008	Cerelose	3.0 gm.

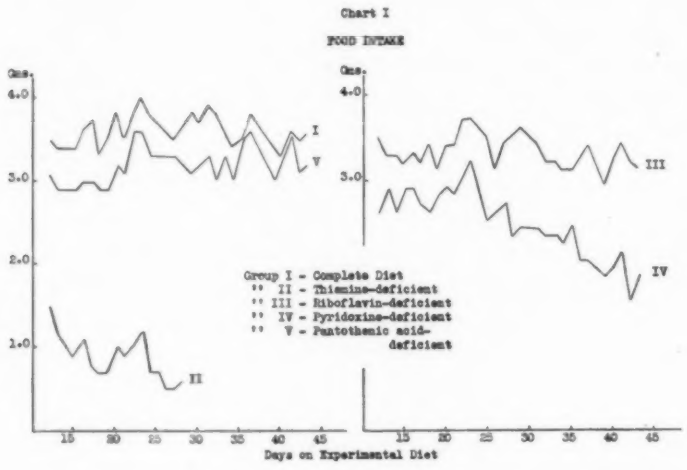
[1] Salts no. 12 (Jones and Foster, '42).

which time again all of the animals of groups II and IV were dead, and two and ten animals were still alive in groups III and V, respectively. The controls (group I) of experiment A were discontinued after 87 days, while those of experiment B were continued for the full 135-day period. The animals were weighed daily for the greater part of the experiment, after which they were weighed but once a week. In the second experiment daily food consumption of each animal was measured from the 12th to the 43rd day. The technique for measuring the food intake was the same as that previously described (Foster et al., '44). The weights of the animals in the corresponding groups in the two experiments were sufficiently similar at each weighing period to warrant combining them. The mice raised on the commercial dog food consisting of natural food materials were slightly heavier at the time the experiments were started

than were the mice raised on the stock diet which is partially composed of purified ingredients. This difference in weight was maintained throughout the experimental period in each of the groups except the control animals (group I). In this case the animals raised on the partially purified diet had attained a weight equal to that of the animals of the corresponding group raised on commercial dog food after approximately 1 month on the experimental diet.

RESULTS

The changes in weight of the animals for various intervals (usually 1 week) are presented in table 2, in which the combined averages of the



weights of males and females of both experiments are given. Table 3 diagrammatically shows whether or not a significant difference in the weights of the animals of any two groups existed at the various weighing dates up to the 50th day. After this there was little or no change in this respect. The growth of the control animals on the complete diet (table 2, group I) was about equal to that of mice of the same age on a stock diet. All of the animals of this group appeared normal and none died. However, one was sacrificed for photographic purposes. In table 2 are also presented the numbers of survivors on each weighing day. In this case the males and females of each experiment are given separately.

The food intake of the animals in experiment B is presented as curves (chart I). There was a definite and approximately uniform difference

between the males and females of each group with respect to growth and food intake. For some unexplainable reason, there was a greater difference between the males and females in both the weights and food intake of the mice on the riboflavin-deficient diet than among any of the other groups. As expected, weights and intake of food of the females of all groups were somewhat less than those of the males.

Thiamine deficiency

The mice on the diet deficient in thiamine gained slightly in weight for a few days, but after about 10 days they lost weight rapidly. The first death occurred on the 19th day, after which the mortality rate was high (table 2, group II). The last animal died on the 31st day. As table 2 shows, there was only a slight difference in the survival times of the animals of experiments A and B. In addition to the marked loss of weight and subsequent death, the animals showed a marked anorexia and certain symptoms which may have been associated with the decreased food intake. Soon after the initial drop in weight the animals became hyperirritable, their backs were arched and their rear legs appeared straight and stiffened. A few days later these mice became generally hypotonic, with a gradually increasing tendency to drag their hind legs. Still later some of them lost their ability to stand and for the most part supported their weights on their bellies, with their legs spread out and rotated inward. At this stage some of them attempted to propel themselves by moving their heads on the floor of the cage. This was followed by a moribund state for a few hours and then death. It is to be noted that in agreement with other investigators, we did not observe in these mice, which were suffering from acute deficiency, the neuromuscular disturbances frequently seen in the rat. The diet used in this case was free of thiamine, but it should be stated that on diets in which the thiamine deficiency was less acute we have frequently observed the same symptoms as those characteristic of the rat. These symptoms may be described as follows: The early irritability and hypertonicity followed by gradual loss of muscular tone are similar to those described above, but before atonia appears there is a tendency for the deficient animal to tilt the head and trunk to one side and to move in circles. This is usually accompanied by a loss of proprioceptive sense and a tendency to fall over the edge of a table when allowed to move about freely. Ocasionally, shortly previous to death, there may be twitching movements of the legs and a retraction of the head or at times the head may be pulled forward.

TABLE 2

Weight data.

DAYS ON EXP. DIET	NUMBER OF SURVIVORS				AVERAGE WEIGHT (GRAMS)[1]
	Exp. A		Exp. B		
	♂	♀	♂	♀	
Group I — Complete					
2	11	9	8	11	14.3 ± .33
9	11	9	8	11	16.0 ± .26
16	11	9	8	11	17.6 ± .25
23	11	9	8	11	18.8 ± .25
30	11	9	8	11	19.9 ± .25
37	11	9	8	11	20.5 ± .25
44	11	9	8	11	20.6 ± .29
50	11	9	8	11	21.0 ± .24
69	11	9	8	11	21.9 ± .30
79	11	9	7	11	22.1 ± .31
Group II — Thiamine deficient					
2	10	10	9	11	14.5 ± .33
9	10	10	9	11	14.7 ± .31
16	10	10	9	10	11.3 ± .21
23	3	8	9	10	9.1 ± .25
30	0	1	1	3	
Group III — Riboflavin deficient					
2	11	9	8	12	15.1 ± .36
9	11	9	8	12	15.7 ± .31
16	11	9	8	12	16.2 ± .30
23	11	9	8	12	16.1 ± .33
30	11	9	8	12	17.0 ± .34
37	11	8	8	12	17.2 ± .42
44	9	8	8	12	17.1 ± .37
50	9	8	8	12	16.8 ± .42
69	5	7	8	11	17.3 ± .48
79	3	7	8	10	17.2 ± .59
98	1	7	7	8	17.0 ± .65
106	0	6	7	7	15.6 ± .49
113	–	6	5	7	16.4 ± .63
120	–	6	4	5	16.4 ± .70

DAYS ON EXP. DIET	NUMBER OF SURVIVORS				AVERAGE WEIGHT (GRAMS)[1]
	Exp. A		Exp. B		
	♂	♀	♂	♀	
Group IV — Pyridoxine deficient					
2	10	10	9	11	14.1 ± .36
9	10	10	9	11	15.6 ± .27
16	10	10	9	11	16.3 ± .28
23	10	10	9	11	16.0 ± .35
30	9	10	8	11	15.9 ± .34
37	7	10	8	10	14.7 ± .47
44	5	7	6	9	13.8 ± .50
50	3	5	6	7	12.5 ± .49
Group V — Pantothenic acid deficient					
2	10	10	9	11	15.1 ± .30
9	10	10	9	11	15.1 ± .22
16	10	10	9	11	16.3 ± .27
23	10	10	9	11	16.4 ± .31
30	10	10	9	11	17.5 ± .31
37	10	10	9	11	17.2 ± .33
44	10	10	9	11	17.6 ± .28
50	10	10	9	11	18.2 ± .33
69	8	10	9	11	18.4 ± .43
79	6	10	8	10	18.5 ± .46
98	5	10	7	9	18.1 ± .40
106	5	10	6	9	17.2 ± .41
113	5	8	4	8	18.3 ± .49
120	5	7	3	8	17.8 ± .52

[1] The weights are given for days after the start of experiment on which there were weight data available in both experiments on significant numbers of animals.

Average weight = the average of the four averages, or the average for animals on a given diet at a given time of the means for each sex for each experiment.

Standard error of the means, which follows the average weight, is computed from the pooled standard deviation measured within each sex and each experiment as follows:

$$\text{A. Pooled } \overline{SD}^2 = \frac{\Sigma d^2_{Am} + \Sigma d^2_{Af} + \Sigma d^2_{Bm} + \Sigma d^2_{Bf}}{N_{Am} + N_{Af} + N_{Bm} + N_{Bf} - 4}$$

$$\text{B. SE of average of 4 averages} = \tfrac{1}{4}\sqrt{\left(\frac{\overline{SD}^2}{N_{Am}} + \frac{\overline{SD}^2}{N_{Af}} + \frac{\overline{SD}^2}{N_{Bm}} + \frac{\overline{SD}^2}{N_{Bf}}\right)}$$

The males of group III of experiment A dropped to 1 on the 98th day, and to 0 on the 106th day; therefore only the females of experiment A were included from this point on. Group V was the only other group carried beyond 98 days. There was no significant difference between the same three units (females of experiment A and males and females of experiment B) in group III and group V, out to the 120th day.

Riboflavin deficiency

The mice on the riboflavin-deficient diet continued to appear normal for about 3 weeks. They not only did not lose weight but actually gained weight at a rate only slightly below that of the mice on the complete diet. The first animal died on the 34th day and thereafter the mortality rate was low (table 2, group III). In the first experiment the last animal died on the 202nd day, and when the second experiment was discontinued on the 135th day, two were still alive. On the average the animals of experiment A died sooner than the animals of experiment B (table 2, group III). This difference appears too large to be the result of mere chance but until repeated, cannot be considered significant. The partially purified stock diet used in experiment A was well supplied with riboflavin as it contained 20% dried grass and 6% yeast.

TABLE 3

Diagrammatic presentation of the significance of the difference in weights among the various groups.[1]

DAY	HEAVY				LIGHT
9	I	III	IV		II
	V				
16	I	IV	V	III	II
23	I	V	III	IV	II

DAY	HEAVY			LIGHT
30	I	V	III	IV
37	I	V	III	IV
44	I	V	III	IV
50	I	V	III	IV

[1] The groups are represented in the order of their weight from heavy to light — left to right. Those in the same block are not significantly different from each other. At the 9th day group V was not significantly different from any of the others.

Of the forty mice on the riboflavin-deficient diet, twenty-three died without showing any specific symptoms. All of the others developed a rather characteristic skin lesion. These lesions made their appearance from the 44th to the 139th day, and although the numbers of animals showing them were about the same in the two experiments, they made their appearance earlier in experiment A (average 58 days) than in experiment B (average 96 days). They were definitely circumscribed, circular areas varying from a pin-point to 1 mm. in diameter and covered with a blood-red, moderately dense scab. The affected area contained from one to a dozen of these scabs. They appeared near the eyes, mouth, top of head or on the tail or ears. Usually, only one or two areas were affected in an individual animal. The ears became most severely damaged. The lesions spread over the surfaces of the ear and the areas became eroded and then necrotic. In some cases the entire

ear sloughed off. The lesions seen in this laboratory are similar to but not identical with those described by Lippincott and Morris ('41–'42).

Day, Darby and Langston ('37) have reported cataracts in rats on a diet deficient in riboflavin. Cataracts were not observed in our mice, but no slit lamp or other special examination was made.

Pyridoxine deficiency

The mice on the pyridoxine-deficient diet grew slowly for 2 or 3 weeks, after which there was a rather marked decline in weight followed by death. The first animal died on the 28th day and the last one on the 67th day. There was practically no difference in survival time of the animals raised on the different stock diets (table 2, group IV). In agreement with Foy and Cerecedo ('41) no specific symptoms were observed. The general decline which preceded death was rather gradual; in some cases the animals lived as long as 41 days after the first signs of general debility were noted. During this period of deficiency, nearly all of the mice excreted a characteristic pigment in the urine, which was noted on the filter papers covering the floor of the cages. No study has been made of this pigment but it stained the paper a brownish-yellow with a slightly reddish tint. The staining was more pronounced and definitely different from that produced by normal urine. Lepkovsky and Nielsen ('42) have described a pigment obtained in the urine of rats on a diet deficient in pyridoxine.

Pantothenic acid deficiency

The mice on the diet deficient in pantothenic acid grew very little but on the other hand, there was very little loss of weight, even during the period of marked symptoms and occasional deaths. The first death occurred on the 67th day. In the first experiment there were four survivors at the end of the experiment (205 days) and in the second experiment (135 days) there were ten survivors. Again there was no real difference in this respect between the animals of experiment A and experiment B (table 2, group V). The symptoms seen were very similar to those described by Woolley ('41) with the exception that the only neuromuscular symptoms observed were profound spasticity of the extremities, acute arching of the spine, and an awkward gait. At no time were tremors, convulsions or paralysis noted. All of the animals showed some loss of hair, varying in degree from a mere thinning of the fur to complete loss except on the head. There was also a marked tendency toward dryness of the skin with the development of scaly

desquamation. A few cases presented areas 5 to 10 mm. in diameter where the skin appeared to have been scratched off. Sixteen of the 40 mice showed hyperemia and edema of the eyelids.

SUMMARY

Using a diet in which the B factors were supplied as pure compounds, a deficiency of each of four of the factors (thiamine, riboflavin, pyridoxine and pantothenic acid) in the albino mouse has been studied.

When thiamine was omitted from the diet the animals lived from 19 to 31 days. They did not show symptoms characteristic of this deficiency as are seen in the rat. In less acute vitamin B_1 deficiency the classical polyneuritic symptoms have been observed in this laboratory.

Mice on the riboflavin-deficient diet lived from 34 to 202 days. Many of them developed characteristic dermatitis about the head and especially on the ears. In some cases nearly the entire ear sloughed off.

No specific symptoms or lesions (other than poor growth and death) resulted from the omission of pyridoxine from the diet. The animals lived from 28 to 67 days. Nearly all of the mice on this diet excreted in the urine a characteristic brownish-yellow pigment.

The first death on the diet lacking in pantothenic acid occurred on the 67th day and there was a survival of 21% when the experiment was discontinued (205 days). A loss of hair was the most outstanding sign of deficiency in these animals. In addition, a number of the mice developed some of the following signs: a profound spasticity of the extremities, acute arching of the spine, awkward gait, dryness of the skin with scaly desquamation, and hyperemia and edema of the eyelids.

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THE NICOTINIC ACID, PANTOTHENIC ACID, CHOLINE AND BIOTIN CONTENT OF FRESH, IRRADIATED EVAPORATED AND DRY MILK

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(Received for publication September 22, 1944)

A number of values for the nicotinic acid, pantothenic acid, choline and biotin content of fresh and processed milk appear scattered through the vitamin literature. Many of the studies, however, contain too few values to permit the estimation of the variability which may occur between different samples of the same type of milk. Moreover, there are only a few studies in which values for fresh and processed milks have been obtained by the same methods and by the same investigators. The present study was undertaken to give us further information on the nicotinic acid, pantothenic acid, choline and biotin content of milk, to outline the variability that might be expected in the content of these factors in milk and especially to determine whether any significant losses occur when evaporated and dry milk are manufactured from fresh milk.

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NICOTINIC ACID

A more thorough investigation of the nicotinic acid content of milk has been made since the place of this vitamin in human nutrition is better understood than that of the other three vitamins and because the method used for the assays appears to be one of the better microbiological methods. In our laboratory the method of Snell and Wright ('41) as modified by Krehl, Strong and Elvehjem ('43) has given excellent

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results as judged by the reproducibility of the data, a minimum of tendency to drift and agreement with results secured by other laboratories.

The results of our microbiological assays for nicotinic acid in milk products are presented in table 1. The fresh milk samples were from Illinois. The evaporated milk samples were from more than twenty different plants well distributed geographically from Maryland to California, and from Mississippi to Wisconsin. The dry milk samples were from Wisconsin, Michigan and Utah. The processed milks were all analyzed within a few weeks after manufacture. The samples were taken during May. The differences found between the various types of

TABLE 1

Nicotinic acid, pantothenic acid, choline and biotin content of fresh, irradiated evaporated and dry milk on a fresh or reconstituted basis.[1]

TYPE OF MILK	NICOTINIC ACID			PANTOTHENIC ACID			CHOLINE			BIOTIN		
	No. of samples	Milligrams per liter		No. of samples	Milligrams per liter		No. of samples	Milligrams per liter		No. of samples	Micrograms per liter	
		avg.	range		avg.	range		avg.	range		avg.	range
Fresh	31	0.91	0.74–1.14	23	3.1	1.9–4.2	10	149	131–169	10	47	32–84
Irradiated evaporated	44	0.98	0.83–1.28	27	3.1	2.4–4.1	6	131	90–208	10	45	29–56
Dry skim	24	0.87	0.72–1.18	16	3.6	3.1–4.3	9	104	54–160	9	34	28–40
Dry whole	4	0.87	0.79–0.91	2	3.3	3.2–3.5	9	142	83–229	9	47	29–58

[1] By multiplying the reconstituted values for evaporated, dry whole, and dry skim milk by 2, 7.7, and 10.3, respectively, they may be converted to an undiluted basis.

milk are not believed to be significant. There is no indication that the processing of either irradiated evaporated milk or dry milk causes any loss of nicotinic acid. Since the heat treatment employed in the preparation of evaporated milk or dry milk is usually more severe than pasteurization, there is little possibility of nicotinic acid destruction by the latter process.

The results presented in table 1 for nicotinic acid agree very well with values found by other investigators. The average value for fresh milk is 0.91 mg. per liter. Snell and Wright ('41) found 0.84 μg. of nicotinic acid per milliliter of milk and Teply, Strong and Elvehjem ('42)

found 0.08 mg. per cent for whole milk, 0.18 for evaporated milk and 0.89 for skim milk powder on an undiluted basis. Noll and Jansen ('41) using a chemical method found 0.6–0.9 μg. of nicotinic acid per milliliter of skim milk.

PANTOTHENIC ACID

The samples used for pantothenic acid assays were obtained during June and July. All processed samples were assayed soon after preparation. The fresh milk samples were from Illinois; evaporated milk samples were from Ohio, Kentucky, Wisconsin, Illinois, Tennessee and Maryland; samples of dry milk were from Wisconsin, Michigan and Utah.

The method used for pantothenic acid assays was that proposed by Hoag, Sarett and Cheldelin ('44). This method uses Lactobacillus arabinosus rather than Lactobacillus casei as the test organism. A more rapid and greater total growth is secured with L. arabinosus and in addition in certain instances the response is perhaps more specific for pantothenic acid than with L. casei. Since the results reported in this paper were obtained without enzymatic treatment or special hydrolysis they represent largely if not entirely free pantothenic acid. However, Atkin, Williams, Schultz, and Frey ('44) report that milk does not contain bound pantothenic acid and data for free pantothenic acid in this product may be fairly indicative of the total content of the vitamin.

Assays were made directly on diluted fresh and dry skim milk. For evaporated milk and dry whole milk a 5% metaphosphoric acid filtrate was prepared. After the protein and fat were separated the filtrate was neutralized before addition to the assay tubes. Direct assays of evaporated, dry whole milk and other milk which had been homogenized consistently gave erratic results which were often 50% or more higher than those obtained with fresh milk. Investigations which cannot be reported in detail here show that the interference which is apparently caused by homogenized milk fat can be eliminated by preparation of the metaphosphoric acid filtrate.

The data on the pantothenic acid content of fresh and processed milk are included in table 1. No significant differences are shown. The range of results indicates a considerable variation in the pantothenic acid content of milk. The average value found for fresh milk, 3.1 μg./ml., compares favorably with the values of 3.2 and 3.3 found by Atkins, Williams, Schultz and Frey ('44). The value of 4.0 μg./ml. found by Strong, Feeney and Earle ('41) is within the range of values reported in table 1.

CHOLINE

The sources of samples for the choline investigation were similar to those for the pantothenic acid assays except that the evaporated milk samples were drawn from a wider range. All the samples were collected during February and March.

The method employed was essentially that of Horowitz and Beadle ('43) using the "cholineless" mutant no. 34486 of Neurospora crassa. The method was slightly modified by the use of 10 ml. of medium and 50 ml. flasks rather than 25 ml. of medium and 250 ml. flasks and by the omission of the permutit exchange. The latter step is included in the original procedure specifically to eliminate methionine, but also probably excludes some other interfering substances. In separate experiments, which will not be reported here, and by calculation, it has been found that the amount of methionine or other interfering substances present in milk is not sufficient to produce significant errors in the assay if the data are taken from the lower portion of the assay curve. If this precaution is observed most of the values are accurate within 10% and probably all are within 20%. Recoveries of choline added to fresh milk ranged from 85 to 102% and averaged 96%.

The average value reported in table 1 for fresh milk, 149 mg. per liter, is in excellent agreement with that found with a chemical method by Engle ('43), namely, 147 mg. per kilogram. The data in table 1 suggest that dry skim milk contains less choline than fresh milk. An explanation for this is the loss of some choline in the phospholipid fraction of the cream.

BIOTIN

For the biotin assays the "cholineless" mutant of Neurospora crassa was used. This organism requires only the two vitamins, choline and biotin, and is well suited for biotin estimations. The wild type of Neurospora crassa should be equally useful but this necessitates the maintenance of a separate stock. One must acknowledge that the specificity for biotin of neurospora and of other test organisms is still open to question until further investigations have been made of compounds which supplant biotin or which antagonize the organism's response to biotin. The wild type of Neurospora crassa responds to desthiobiotin as has been shown by Lilly and Leonian ('44). If this compound should be present in milk, the values in table 1 would reflect its presence as additional biotin.

The sources of the samples used for the biotin investigation were similar to those for choline. The samples were obtained during February, March and April.

The figures for biotin presented in table 1 are somewhat misleading. The presence of a few high values in the fresh, irradiated evaporated and dry whole milk series widened the range of the results and markedly raised the average. Most of the values fell between 30 and 40 μg. per liter and thus were in good agreement with the 30–40 mμg. per milliliter (30–40 μg. per liter) reported by Lampen, Bahler, and Peterson ('42), who used Cl. butylicum as the test organism. No high values were encountered in the dry skim milk series and this is reflected in the lower average.

SUMMARY

1. The average nicotinic acid, pantothenic acid, choline and biotin content of fresh milk was found to be 0.91 mg., 3.1 mg., 149 mg. and 47.1 μg. per liter, respectively.

2. In the processing of irradiated evaporated, dry skim or dry whole milk, the data show no significant losses in the contents of nicotinic acid, pantothenic acid, choline and biotin with the possible exception of the slightly lower biotin content of dry skim milk.

3. The possibility of small and probably insignificant processing losses cannot be definitely excluded because the raw milk sources of the different types of milk tested were not identical, because of the variability of the data, and because of the limitations in accuracy of the microbiological methods used.

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THE EFFECT OF SUCCINYLSULFATHIAZOLE AND PHTHALYLSULFATHIAZOLE ON THE BACTERIAL FLORA OF RAT FECES

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The feeding of sulfonamides that are poorly absorbed from the intestinal tract [e.g., sulfaguanidine, succinylsulfathiazole (SST) and phthalylsulfathiazole (PST)] causes a decrease in the coliform count in the feces of the dog (Poth et al., '42), rat (Gant et al., '43), monkey (Welch et al., '42), mouse (White, '42) and man (Marshall et al., '40). Despite this decrease, the total number of organisms in such feces is reported to remain relatively constant (Light et al., '42), or actually to increase (Gant et al., '43). The prolonged feeding of such sulfonamides in highly purified diets results, in the rat, in the development of signs of deficiencies (Welch and Wright, '43; Black et al., '42) that are not seen when the purified diets are fed without these sulfonamides, or when the drugs are added to ordinary commercial rations.

It has been suggested (Black et al., '41) that these signs of deficiency are caused by the inhibitory action of the drug on vitamin-synthesizing bacteria in the intestine, and that Escherichia coli, in particular is the organism so affected. In this connection Gant et al. ('43) suggested that, even though the coliform count of rats continued on the drug-diet returns to normal, such organisms represent sulfonamide-resistant strains that have lost the ability to synthesize the growth factors involved.

In view of these reports it seemed advisable to study the influence of diet on the fecal flora of rats, and to investigate the vitamin-synthesizing powers of a sulfonamide-resistant organism.

EXPERIMENT I

Techniques. A fecal pellet for bacteriological examination was gently expressed from the rectum of the rat, and was allowed to fall into a

sterile petri dish. Using aseptic techniques, the pellet was transferred to a graduated centrifuge tube and emulsified in saline solution with a swab stick. After dilutions of the original suspension were made, the tube of emulsified feces was centrifuged for 20 minutes at 1700 revolutions per minute, and the approximate volume of solid material was measured. It was assumed that 1 ml. of solid was equivalent to 1 gm. (wet weight) of feces, and on this basis the dilutions of the fecal material were calculated.

Suitable amounts were transferred routinely from the dilution tubes to Durham tubes of bromcresol purple-lactose broth and to tubes of freshly steamed thioglycollate medium. Occasionally plates were made with nutrient agar or desoxycholate agar. After these inoculations were made, the dilution tubes sometimes were held at 80°C. for 10 minutes to destroy the vegetative forms of bacteria. A second set of thioglycollate tubes then was inoculated. Tubes and plates were incubated at 37°C., aud were read after 72 hours. All media used contained p-aminobenzoic acid (0.001%).

The number of aerobic organisms present in a sample was calculated from the lactose tube of greatest dilution that showed growth. The production of acid and gas in the lactose tubes was taken as presumptive evidence of the presence of coliform organisms. As confirming evidence, streaks on desoxycholate plates were made from the two highest dilution tubes showing such acid and gas, and the plates were examined after 24 hours for the presence of typical coliform colonies. Growth in the bottoms of the thioglycollate tubes was used as a measure of anaerobic organisms; similar growth in the second set of thioglycollate tubes (those inoculated from the heated dilutions) was used as a measure of the presence of anaerobic spores in the original material.

Effects of succinylsulfathiazole. Six 50-day-old piebald rats from the Sunny Hill strain were placed in individual cages with wide-meshed screen floors and fed stock ration [1] and water ad libitum. Table 1 includes the average total counts of "aerobes" (including coliform bacteria), "anaerobes," and coliforms in the feces of these rats. It should be mentioned that the figures in this table average not only the variation which occurred among the six rats, but also include the normal fluctuation always observed from count to count for any one rat. Counts on one of these rats were continued for 146 days, during which time the average number of aerobic organisms was 10^8; anaerobes, 10^7; coliforms, 10^5; and anaerobic spores, 10^5 per gram of feces.

[1] Purina chow.

After 21 days on the stock ration, five of the rats described above were placed on a highly purified diet (table 2). Within a day the fecal pellets changed from the more bulky, brown, and softer state characteristic of the animals given the stock ration to a blue-green, compact mass. Almost all the fecal samples showed a drop in each of the three counts on the first or second day; this was followed by a gradual return to higher levels. Table 1 shows the results of these counts over a period of 15 days.

TABLE 1

Average counts of bacterial flora of rat feces. Effect of age and diet on the rat.

DIET	AEROBIC ORGANISMS		ANAEROBIC ORGANISMS		COLIFORM ORGANISMS		ANAEROBIC SPORES	
	Exp. I [1]	Exp. II [2]	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
Succinylsulfathiazole tests								
Stock	10^9(35)	10^9(9)	10^8(25)	10^8(8)	10^7(34)	10^8(8)	10^3(3)	10^4(8)
Purified	10^8(19)	10^8(11)	10^7(21)	10^8(6)	10^6(23)	10^6(11)	10^4(4)	10^3(6)
0.5% SST		10^8(8)		10^8(6)		$<10^3$(5) [3,4]		10^3(6)
2% SST	10^8(39)	10^7(6)	10^9(39)	10^7(4)	10^3(41)	$<10^3$(4) [4]	10^3(10)	10^3(4)
Phthalylsulfathiazole tests								
Purified	10^8(17)		10^8(17)		10^7(17)		10^3(2)	
0.5% PST	10^8(21)	10^7(4)	10^8(21)	10^7(3)	10^3(21)	$<10^3$(3) [4]	10^3(5)	10^3(3)
1% PST	10^8(19)		10^9(19)		$<10^3$(19)		10^3(3)	
2% PST	10^8(20)	10^8(6)	10^9(20)	10^8(4)	$<10^3$(20)	$<10^3$(6)	10^3(5)	10^3(4)

Counts are given as per gram of feces (wet weight).

Figures in parentheses indicate the number of counts included in the average.

[1] In experiment I the rats were about 75 days old when placed on the succinylsulfathiazole diets, while the rats given phthalysulfathiazole were about 40 days old when placed on the drug diet.

[2] In experiment II all the rats were about 60 days old when placed on drug diets.

[3] The counts for one rat in this group remained above this average.

[4] After 3 days on the drug diet.

Two of these rats were continued on this purified diet for 132 days. Average fecal counts of these animals during this period were: aerobes, 10^8; anaerobes, 10^7; coliforms, 10^6; and anaerobic spores, 10^4 per gram of feces.

At the end of the above 15-day period three of the rats on the purified ration were placed on a diet in which 2% SST replaced an equal amount of sucrose. The slight diarrhea which Gant et al. ('43) observed when SST was added to a purified diet has not been seen in this laboratory, probably because "Cellu flour" was incorporated in the basal diet. Although no change in the physical characteristics of the fecal pellets

occurred, there was an immediate drop in the coliform count. Following this initial drop, considerable variation was noted in the coliform counts of these rats: for one animal such variation was quite small and the average value was 10^5 per gram of feces; counts on another animal fluctuated widely (0–10^6) and averaged 10^3 coliform organisms per gram; feces from the third rat were almost entirely devoid of coliform organisms, after the addition of the drug to the diet, until near the end of the experiment, when the count gradually rose to 10^4 per gram. Counts over a period of 119 days are given in table 1.

TABLE 2

Composition of the purified diet.

INGREDIENTS	PARTS	CONTENT OF VITAMIN MIXTURES	
			gm.
Sucrose	61.78	Vitamin A D E concentrate	
Casein (vitamin free)	18.00	(450,000 U.S.P. units A	
Primex vegetable oil	10.00	and 90,000 U.S.P. units D	
Salt mixture (Hubbell,		per g.)	7
Mendel and Wakeman, '37)	4.00	a-Tocopherol	2
Cellu flour	4.00	Corn oil	41
Corn oil	2.00		
Choline chloride	0.10	Vitamin mixture:	
Vitamin A D E concentrate	0.10	Inositol	2.0
Vitamin mixture	0.0236	Ca pantothenate	1.1
Vitamin K (2-methyl		p-Aminobenzoic acid	1.0
1–4 naphthohydroquinone		Nicotinic acid	1.0
diacetate)	0.0010	Riboflavin	0.4
		Pyridoxine HCl	0.2
Total ingredients	100.0046	Thiamine HCl	0.2

Reference to table 1 shows that the differences in the counts of the total aerobic and anerobic organisms, and of the anaerobic spores found in the feces of rats on these three diets, is at most a hundred fold and therefore is probably insignificant. However, the drop in the number of coliform organisms found in the feces of the rats changed to the diet containing 2% SST is significant.

Since the rats used in this experiment were relatively old when they were placed on the drug diet, deficiency symptoms characteristic of a 2% SST diet (Welch and Wright, '43) were slow in developing. The first noticeable symptom was a tendency for the eyelids to stick together; this was observed for one of the rats on the twentieth day of the diet. Later, spectacled eye, thinning of the hair on the head, loss of weight, slight graying of the hair, sore mouth, and porphyrin-caked whiskers were noted.

Starting on the 105th day of the drug diet, two of the three rats receiving this ration were given daily subcutaneous injections of a supplement containing folic acid concentrate [2] equivalent to 25 μg. of potency 40,000 material (Mitchell and Snell, '41) and 5 μg. of crystalline biotin. The drug diet and injections were continued for a period of 14 days. During this time the two supplemented rats improved in physical appearance (condition of eyes, whiskers, and hair) and were relatively constant in weight. Because these rats were essentially full grown, a marked increase in weight could not be expected. In the case of the unsupplemented rat receiving the drug diet, the deficiency symptoms became increasingly severe.

Fecal samples were collected and examined during this time. No appreciable changes were observed in the counts. This indicated that the curative effects of folic acid and biotin were brought about by replacement therapy and not by increasing the number of coliform organisms in the feces.

Immediately before and at the end of the period of supplemental feeding total leucocyte counts were made on blood from each of the three rats on the drug diet. Before supplementation all three rats had low counts (4,600–5,580). At the end of the experiment, the blood of the two supplemented rats showed normal counts (13,300; 17,100), while the count of the unsupplemented rat was still low (6,830). This confirms the work of Daft and Sebrell ('43), who reported the successful treatment with folic acid of the leukopenia which had developed in rats fed SST in a purified diet.

Twenty-four-hour fecal samples were collected from rats on each of the three diets described above. The pellets from a single rat were ground in a mortar and divided into two approximately equal samples, each of which was weighed and placed in a small pyrex bottle. One of the two samples was covered with 10 ml. of 6N sulfuric acid and then autoclaved at 15 pounds pressure for 1 hour. After cooling, the autoclaved sample was neutralized, diluted, and filtered. Suitable dilutions were made of the filtrate, and these were assayed for biotin by the microbiological method of Landy and Dicken ('42).

To the second sample of ground feces were added takadiastase (2% of the weight of the feces), 20 ml. of water, and 2 ml. of benzene. The bottle was tightly stoppered, and the mixture was allowed to digest at 37°C. for 24 hours, after which it was autoclaved for 15 minutes at 15

[2] This concentrate was prepared from grass juice powder by Dr. L. D. Wright of these laboratories. The procedure used was similar to that described by Hutchings, Bohonos and Peterson ('41), and was carried through the superfiltrol eluate stage.

pounds pressure. After dilution, the sample was filtered, and the filtrate was assayed microbiologically for folic acid and pantothenic acid according to the method of Landy and Dicken ('42). The folic acid standard used in these tests was a concentrate containing 200 μg. of folic acid per gram.[3]

The results of these assays are shown in table 3. In almost all cases the feces from rats on the drug diet contained appreciably smaller amounts of biotin and folic acid than were found in the feces of rats on the stock and on the purified rations. Comparison of figures for the rats on the stock and the purified diets showed that, except for pantothenic acid, the greater daily elimination of the vitamins by the rats fed the stock diet was caused only by the larger quantity of feces

TABLE 3

The effect of diet on the fecal excretion of biotin, folic acid, and pantothenic acid by the rat.

DIET	BIOTIN		FOLIC ACID		PANTOTHENIC ACID	
	μg./gm.	μg./day	μg./gm.	μg./day	μg./gm.	μg./day
Stock	0.91(5) [1]	8.6 (5)	5.5 (7)	42.7(7)	62 (6)	428 (6)
Purified	0.60(6)	0.78(6)	4.7 (9)	5.3 (9)	29.8(9)	35.4(9)
0.5% PST	0.09(4)	0.15(4)	0.36(5)	0.54(5)	17.9(5)	26 (5)
1% PST	0.08(3)	0.08(3)	0.59(4)	0.65(4)	12 (4)	10 (4)
2% PST	0.07(3)	0.13(3)	0.9 (4)	1.2 (4)	17 (4)	18 (4)
2% SST	0.28(4)	0.39(4)	2.5 (6)	4.2 (6)	16.9(6)	27.2(6)

[1] Figures in parentheses indicate the number of assays included in the average.

excreted on this diet; the folic acid and biotin contents per gram of feces remained essentially unchanged.

In this connection it is of interest to comment on the relative vitamin contents of the diets used. According to Taylor et al. ('42b) Purina Chow has the following approximate composition: pantothenic acid, 14 μg./gm.; biotin, 0.19 μg./gm.; and folic acid, 0.71 μg. of potency 40,000 material per gram. The purified diet, which was also the base for the drug diet, contained neither biotin nor folic acid except for possible traces associated with purified materials, but did contain 44 μg. of pantothenic acid per gram of diet. In spite of this greater pantothenic acid content, much less of this vitamin was excreted per gram of feces by the rats on the purified diet than by the rats on the stock ration.

Effects of phthalylsulfathiazole. A number of 38-day-old rats of the Rockland strain were divided into four groups. Group I received the

[2] Kindly provided by the Lederle Laboratories.

purified diet; group II, the purified diet containing 0.5% PST; group III, the purified diet containing 1% PST; and group IV, the purified diet containing 2% PST. The rats in group IV did not show normal weight gains, nor was their physical appearance good. Several from this group died before the end of the experiment.

The outstanding effect of the addition of PST to the diets was a definite decrease in the number of coliform organisms present in a given weight of feces. This is shown in table 1 in which counts taken over a period of 77 days are shown. From the rats fed the two higher drug levels the coliform counts became very low within the first week after the addition of the drug to the diet, and remained low throughout the entire experiment. Although a definite reduction in the number of coliform organisms occurred in the feces of rats fed diets containing 0.5% PST this reduction was not maintained consistently. The count varied from 0 to 10^4 coliforms per gram of feces.

Beginning on the fifty-sixth day of the experiment, a rat from group II (0.5% drug) and one from group IV (2% drug) were each given, by stomach tube, daily supplements of 5 μg. of crystalline biotin and folic acid concentrate equivalent to 25 μg. of potency 40,000 material. Both rats showed an immediate improvement in physical appearance and a marked gain in weight. The supplemented drug diet was fed for 3 weeks, during which time there was no appreciable change in the bacterial counts of the feces of either rat.

In addition to the bacterial counts made on the feces of rats fed these diets, occasional microbiological assays also were conducted to measure the amounts of biotin, pantothenic acid, and folic acid eliminated in the feces. Twenty-four-hour samples were collected and treated as outlined above for the rats given SST. Appreciably smaller amounts of biotin and folic acid were excreted by rats receiving the drug-containing diets. Table 3 shows average results obtained from these assays.

EXPERIMENT II

Data from experiment I showed that the fecal coliform counts of the young rats fed PST were reduced to a lower level than were the coliform counts of the older rats which were fed SST. A second experiment was set up using 50-day-old rats of the Albino Farms strain to determine whether the difference in the ages of the rats used in experiment I had any influence on the results obtained. Bacterial counts for experiment II are included in table 1. Here the 2% level of either drug reduced the fecal coliform counts to a low figure. This reduction, however, occurred

more rapidly when PST was used than when an equivalent percentage of SST was included in the diet.

In this experiment the animals were maintained on the stock ration for 3 days after which time all but three of the rats were fed the purified diet for a period of 5 days. Both 0.5 and 2% levels of PST and of SST were then added to the purified diets of some of these rats. Supplementation of the diet with folic acid and biotin was not carried out in this experiment.

The bacteriological techniques used were as described for experiment I except that 0.5% dextrose-beef heart infusion broth tubes were inoculated and incubated in Weiss-Spaulding jars for the determination of the anaerobic and anaerobic spore counts.

EXPERIMENT III

While experiments I and II were in progress, a laboratory strain of Escherichia coli was made resistant to sulfonamides by daily transfers in bacto-peptone broth containing gradually increasing amounts of sulfanilamide (SA). When growth studies showed the strain to be resistant to M/100 SA (in bacto-peptone medium), a comparison was made of the amount of folic acid found per milliliter of culture of the resistant and of the parent strain. The folic acid standard used in these assays was a solution of crystalline folic acid.[4]

When the tests were run in bacto-peptone medium containing no sulfonamide, the parent strain developed, in 18–24 hours, a turbidity of 257–375 units, as measured by a Klett-Summerson photoelectric colorimeter, and assays by Streptococcus fecalis (the so-called Streptococcus lactis R strain) showed 0.018 to 0.026 μg. of folic acid per milliliter of culture. Under the same conditions, the resistant strain developed a turbidity of 214–260 units, with 0.0098–0.0196 μg. of folic acid per milliliter of culture.

When the test flasks contained M/100 SA in the medium, and large inocula were used (1 ml. per 20 ml. of medium, as was also used above), the parent strain developed a turbidity of 208–241 units after 18–24 hours growth, and 0–0.0004 μg. of folic acid was found per milliliter of culture. Under similar conditions, the resistant strain showed a turbidity of 300–400 units, and produced 0.0016–0.0051 μg. of folic acid per milliliter of culture. Adequate controls were run to correct for the amount of folic acid present in the uninoculated medium, and to demonstrate that the amount of sulfonamide used had no inhibitory effect on the assay organism.

[4] Lederle Laboratories.

These results indicate that the presence of the sulfonamide in the medium, and not the state of sulfonamide resistance of the organism, determines the amount of folic acid produced. Details of the experiments quoted here, and of two tests using sulfathiazole as the sulfonamide, are given in table 4.

TABLE 4

Folic acid synthesis by a sulfonamide resistant and a sulfonamide sensitive strain of Escherichia coli.

TEST NO.	MEDIUM	HOURS INCUBATION	SENSITIVE STRAIN		RESISTANT STRAIN	
			Turbidity	Folic acid	Turbidity	Folic acid
				μg./ml. culture		*μg./ml. culture*
I	BP [1]	24	355	0.026	220	0.0098
II	BP	24	375	0.024	260	0.0128
III	BP	24	305	0.018	216	0.0093
IV	BP	18	257	0.020	315	0.0196
V	BP	18	289	0.019	214	0.0173
I	BPSA [2]	24	230	0.0002	350	0.0016
II	BPSA	24	226	0.0004	330	0.0051
IV	BPSA	18	208	0.0003	397	0.0051
V	BPSA	18	241	0.0000	400	0.0047
IV	BPST [3]	18	142	0.0002	180	0.0023
V	BPST	18	137	0.0000	213	0.0060

[1] BP = Bacto-peptone medium.
[2] BPSA = Bacto-peptone medium containing M/100 sulfanilamide.
[3] BPST = Bacto-peptone medium containing M/2000 sulfathiazole.

DISCUSSION

Bacteriological examination of the feces of rats showed that the number of organisms present varied from rat to rat and in the same animal from day to day. Changing the diet of the rat from Purina Chow to a highly purified diet containing adequate amounts of all the known growth factors did not change significantly the bacterial flora of the feces. However, when either SST or PST was added to such a purified diet, a noticeable drop in the number of coliform organisms in the feces occurred. No significant increase or decrease was observed in the number of aerobes present; presumably the decrease in the number of coliform organisms was balanced by an increase in other organisms.

In experiment I, when the drug included in the purified diet was SST (2%), the coliform counts varied over a rather wide range. Concentrations of PST at 1 or 2% of the diet proved much more effective in reducing the fecal coliform count. In this experiment, the rats receiving

PST were younger and of a strain differing from those receiving SST. In the experiment in which all the rats used were of the same strain and age, the 2% SST diet reduced the coliform count to as low a level as did the 2% PST diet, but 0.5% PST was more effective in reducing this count than was 0.5% SST.

Rats fed purified diets containing either drug developed, in time, symptoms usually associated with dietary deficiencies. The growth and weight gains were subnormal; thinning of the hair occurred on the head and, in some cases, on the abdomen; the coat became dull and dry; porphyrin-caked whiskers developed. In the longer experiment (I), especially in the rats fed SST, spectacled eye and slight graying of the hair were observed, and patches of black hair at the base of the fore-legs turned a deep chocolate brown in color. The supplemental feeding of biotin and folic acid resulted in a greatly improved physical appearance. Blood counts on the SST-fed rats showed a definite improvement in the total leucocyte count after such a period of vitamin feeding. The drug was not removed from the diet during the period of supplementation, and no change in the number, kind, or distribution of fecal organisms was noted.

Assays of 24-hour fecal samples from experiment I to determine the amounts of biotin, folic acid, and pantothenic acid excreted gave rather irregular results. Increasing amounts of biotin seemed to be excreted with the feces as the rat grew older. This is more clearly apparent in the older rats fed SST than in the younger rats fed PST, and may merely reflect the increase in the proportionate size of the cecum as the rats grew larger (Taylor et al., '42a). For folic acid and pantothenic acid, the limits of variation of vitamin per gram of feces, or of vitamin per day were rather wide, and this variation did not seem to have any relation to the age or diet of the rat. Animals fed the SST or PST diet excreted significantly lower amounts of biotin and folic acid than did those not receiving the drugs. Pantothenic acid levels in the feces of rats fed drug diets appear to be depressed.

Counts of the intestinal flora, as performed in these experiments, indicate that a more complete survey of the intestinal organisms might be of value in following the effects of these and similar drugs on fecal organisms, and perhaps would lead to a better understanding of the action of such compounds.

SUMMARY

When a highly purified diet containing 0.5% to 2% succinylsulfathiazole or phthalylsulfathiazole was fed to rats over a long period of time

there developed signs of nutritional deficiency which were corrected by the feeding of biotin and folic acid. The feeding of such sulfonamides also caused a decrease in the coliform count of the rat feces, but caused no significant change in the number of "total aerobes," "total anaerobes," or anaerobic spores in the feces. Lower levels of biotin, folic acid and pantothenic acid were excreted in the feces of rats fed diets containing the drugs than were excreted by rats fed the same diet without the drug.

Neither a sulfonamide-resistant nor a sulfonamide-sensitive strain of Escherichia coli synthesized as much folic acid when grown in the presence of sulfonamides as was synthesized during growth in a medium not containing the drug.

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